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The longterm goal of our work is to elucidate the pattern of expression of the five somatostatin receptor (SSTR1-5) isoforms in breast cancer, to determine which SSTR subtypes and signalling mechanisms mediate the antiproliferative effects of somatostatin (SST), whether the available SST analogs are effective in binding to these antiproliferative SSTR subtypes, and whether the pattern of SSTR expression in tumors can provide an independent prognostic marker. Towards these goals, we have characterized the pattern of expression and relative abundance of mRNA and proteins for SSTR1-5 in 101 primary breast cancers. All tumors expressed at least one SSTR and the majority expressed multiple SSTRs including subtypes 2, 3, and 5 which bind to octapeptide SST analogs. A good correlation was found between SSTR protein expression by immunocytochemistry and mRNA analysis by RT-PCR. SSTRs exert significant antitumor activity both by cytostatic and cytotoxic (apoptotic) actions in breast cancer cells. Apoptosis is dependent on estrogen receptor expression and is potentiated by tamoxifen. Apoptosis is induced uniquely via the SSTR3 subtype and is associated with induction of wild type p53 and of Bax and endonuclease 2. By contrast, the other four SSTR subtypes signal cell cycle arrest with the following rank order - SSTR5 > SSTR2 > SSTR4 > SSTR1. These changes are associated with the induction of the retinoblastoma protein pRB and the cyclin-dependent kinase inhibitor p21.

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INTRODUCTION

The neuropeptide somatostatin (SST) exerts potent biological effects which include inhibition of hormone secretion, modulation of cognitive, motor, and sensory function, and inhibition of cell proliferation. These actions are mediated by a family of G protein coupled receptors with currently five members termed SSTR1-5. The ability of SST to block hormone secretion led to the application of longacting SST analogs such as Octreotide (OCT) in the early 1980's for the treatment of hormone oversecretion from pancreatic, intestinal, and pituitary tumors. A number of subsequent developments followed which have led to mounting interest in the oncological utility of SST compounds for many different forms of cancer including breast, prostate, colon, and small cell lung cancer. These include:

(1) the discovery that common solid tumors derived from mammary, gastric, colorectal, pancreatic, lung, prostate, and cervical tumors are rich in SSTRs;
(2) shrinkage of pituitary and intestinal tumors with OCT treatment;
(3) potentiation of the antineoplastic effect of tamoxifen (TAM) and ovariectomy by OCT in experimentally induced rat mammary carcinoma *in vivo*;
(4) the discovery of a direct tumoricidal effect of SST analogs on tumor cell lines;
(5) the ability to visualize and monitor SSTR positive tumors by *in vivo* receptor scintigraphy;
(6) the possibility of selective ablation of SSTR positive tumors and their metastases by targetted radiation or targetted cytotoxic agents using SST radioligands.

These observations have sparked great interest in the oncological properties of SST and led to the approval of multi-center clinical trials by the NSABP and the NCI Canada to look at the effects of SST with or without tamoxifen in stage I and stage II breast cancer.

The antiproliferative effects of SST are mediated -

(1) directly by SSTRs present on tumor cells which inhibit mitogenic signalling of growth factor receptor kinases and result in growth arrest and/or apoptosis.
(2) indirectly via SSTRs present on nontumor cell targets to block the secretion of hormones and growth factors which promote tumor growth, and to inhibit angiogenesis, promote vasoconstriction, and modulate immune cells.

Several SSTR subtypes and signal transduction pathways have been implicated in mediating the antiproliferative effects of SST. Most interest is focused on a SST sensitive PTP shown to dephosphorylate and inactivate growth factor receptor kinase activity. By studying SSTR1-5 individually transfected in CHO-K1 cells, three studies have reported that subtypes 1, 2, and 5 reduce cell growth, subtypes 1, 2 acting via a PTP and subtype 5 through inhibition of the IP3/Ca^{2+} pathway. Recently, 66 kDa SH2 domain containing PTP called SHP1 ((PTP1C/HCP/SHPTP1) has been shown to associate with membrane SSTRs upon SSTR activation. The effect of SST on cell cycle progression and apoptosis was first described in MCF-7 cells and subsequently in AtT-20 pituitary cells. The SSTR subtype selectivity of this response as well as the signalling pathways involved remain to be defined.

Most tumors studied express variable levels of SSTRs as assessed by autoradiography and membrane binding analyses. By autoradiography, there is a rich expression of SSTRs in peritumoral veins surrounding poorly differentiated tumors. There are currently no pure SST agonists for any of the individual SSTR subtypes. Thus, binding analysis using standard radioligands although important do not tell us about the pattern of expression of individual SSTR subtypes. RT-PCR analysis of mRNA for SSTR1-5 has shown that normal tissues such as the pituitary as well as most tumors express multiple SSTR genes. In the case of breast cancer, 15-66% of primary human breast tumors are SSTR positive by binding analysis. SSTR expression is positively correlated with tumor size and inversely correlated with EGF receptor levels and tumor differentiation. 75% of breast cancers are receptor positive by *in vivo* receptor imaging with an Indium label OCT analog. Expression of SSTR mRNA in breast cancer has been reported by Vikic-Topic et al from the Mayo Clinic and by our group. The Mayo Clinic series used simple RT-PCR (without Southern blots) for analyzing 44 primary breast cancers for SSTR1-4. They found SSTR2 in all but one sample with lesser expression of transcripts for the remaining subtypes SSTR1 > SSTR3 > SSTR4. The pattern of SSTR expression was independent of patient age, histological grade, and levels of estrogen and progesterone receptors. In a preliminary study of the analysis of all five SSTR mRNAs, we reported that SSTR5 was the predominantly expressed subtype and have since completed a semiquantitative analysis of SSTR1-5 mRNA in over 100 samples as described in this report.

Whilst the precise function of SSTRs in tumors is still unclear, their presence can be exploited for use as diagnostic, prognostic, and therapeutic targets. All oncological studies with SST to date have used only the OCT analogs. This means that any effects mediated via SSTR1 and 4 subtypes have been missed. In addition, since tumors express multiple SSTR subtypes some of which may be involved in the antiproliferative effects, the ideal approach for diagnosis and therapy of tumors is to characterize the pattern of expression of SSTRs in tumors, determine the subtypes involved in the antiproliferative effects of SST and target the subtypes with appropriate agonists. **It is our underlying hypothesis that SST inhibits the growth of breast cancer by acting both directly on tumor cells as well as on surrounding nontumor cells, that these effects are mediated by distinct SSTR subtypes which are differentially expressed on these cells and differentially regulated by estrogens and antiestrogens. We hypothesize that SST modulation of PTP is a key step in the signalling cascade leading to inhibition of growth as well as induction of apoptosis, and that one such PTP (SHP1) is recruited from the cytosol to the plasma membrane in a subtype selective manner.**

LONGTERM OBJECTIVES

The longterm goal of our work is to elucidate the pattern of expression of the five individual SSTR subtypes in breast tumor, to determine whether their pattern of expression can provide an independent prognostic marker, and whether the SSTRs are modulated by estrogens and antiestrogens. In addition, we wish to determine the subtype selectivity for the antiproliferative effects of SST as well as the role of SHP1, p53, and other downstream effectors in mediating the cytostatic and cytotoxic effects of SST. Towards these goals we have made significant progress towards the completion of the major tasks scheduled for year one.

SPECIFIC TASKS PROPOSED FOR YEAR 1:

Dr. Y.C. Patel

1) Expression of SSTR1-5 mRNA in tumor samples by RT-PCR
2) Expression of SSTR1-5 mRNA by *in situ* hybridization analysis
3) Analysis of tumor cell lines for immunoreactive somatostatin and SSTR mRNA
4) Regulation of SSTR1-5 by estrogens/tamoxifen

Dr. C.B. Srikant

1) Studies correlating SSTR binding with PTP-1C regulation and growth inhibition
2) Studies of subtype selective SSTR association with PTP in CHO-K1 and COS-7 cells
3) Studies of SSTR subtype selectivity for apoptosis
4) Elucidation of the role of PTP in SSTR signalled apoptosis

With the rapid availability of the large batch of tumor samples from the Manitoba Breast Tumor Bank as well as a successful characterization of our panel of SSTR antisera against all five subtypes, we concentrated our efforts entirely on RT-PCR analysis and receptor immunocytochemistry. A lower priority was assigned for the *in situ* hybridization work and now that we have excellent working antibodies, we will decide in year 2 if we need to conduct the *in situ* hybridization work after we see the complete results of our immunocytochemistry.

METHODS AND RESULTS

(I) ***Expression of SSTR1-5 mRNA in human breast cancer tissue.***

Samples: 110 samples of surgically removed primary human breast cancer tumors were received in two batches from the Manitoba Breast Tumor Bank. Samples were obtained as 4 x 20 μM frozen sections per tumor. Adjacent sections were characterized histologically for (i) tumor grade (Nottingham Scale, 3,4,5 = well differentiated; 6, 7 = moderately differentiated; 8, 9 = poorly differentiated); (ii) percent invasiveness (invasive tumor component); (iii) tumor size (in cm); (iv) lymph node status (positive or negative); (v) estrogen receptor status (fmol/mg protein, ligand binding assay, < 3 = negative; > 3 = positive); (vi) progesterone receptor status (fmol/mg protein, ligand binding assay, < 10 = negative; 10-5 = borderline; > 15 = positive). Nine samples could not be processed adequately because of insufficient sample, degraded RNA or loss of sample during processing. A total of 101 samples comprising 91 ductal NOS tumors (Table 1) and 10 tubular/lobular/mixed tumors (Table 2) were analysed for SSTR1-5 mRNA.

Analysis of SSTR1-5 mRNA by RT-PCR: Total RNA was extracted by the acid guanidinium thiocyanate phenolchloroform method, treated with DNAse and reverse transcribed. The cDNAs obtained were amplified by PCR using primers and incubation conditions described

by Panetta and Patel (Life Science 56:333, 1994). Actin mRNA was concurrently amplified as an internal control. The reaction mixtures were analysed by electrophoresis followed by Southern blots probed with ^{32}P labelled genomic SSTR DNA or cDNA. The intensity of the hybridization signal was quantitated densitometrically and expressed as a ratio of the actin signal.

Results: We were able to analyse 82 of the 91 ductal NOS tumor samples for all five SSTR mRNA. The analysis was incomplete in 9 samples because of insufficient mRNA or for technical reasons. The 82 samples that were completely analysed were all positive for SSTR mRNA expression. Indeed 32 of the 82 samples (39%) were positive for all five SSTR mRNA. 25 (30%) expressed 4 subtypes, 16 (20%) expressed 3 SSTR subtypes, and 9 (11%) were positive for two subtypes. Thus, all tumors in this series expressed more than one SSTR isoform. Of the individual SSTR subtypes, hSSTR5 was the predominant receptor being expressed in 80/89 (90%) samples analysed. SSTR4 was expressed in 76/86 (88% of samples), SSTR3 in 77/90 (86%) of samples, SSTR2 in 62/89 (70%) of samples, and SSTR1 in 54/87 (62%) of samples. Not only was SSTR5 the most frequent receptor subtype found in these tumors but its mRNA level was also the most abundant (8.3 ± 0.58 fold that of actin mRNA). The relative amounts of mRNA for the other four subtypes was comparable to each other and ~ 4-5 fold that of actin. In the case of SSTR1 and SSTR2, however, since 30-40% of these tumors were negative for these receptor mRNAs, this means that those tumors that were positive expressed relatively high levels of mRNA. No correlation was found between SSTR expression, tumor size, lymph node status or ER/PR status in this series. However, there was a trend towards lower total SSTR expression in high grade tumors and higher expression in low grade tumors. We are still accruing additional samples and have, therefore, deferred a complete statistical analysis until the sample size is completed.

The small group of tubular/lobular/mixed cancers (Table 3) were also all positive for SSTR expression. Nine of these tumors expressed multiple subtypes, three being positive for all five. SSTR4 was the most frequently expressed subtype followed by SSTR5, SSTR2, SSTR3, and SSTR1. These tumors were also rich in SSTR5 mRNA with a mean mRNA level 10.7 fold greater than that of actin. mRNA for SSTR1-4 was expressed in approximately equal abundance (~ 3 fold that of actin) (Table 4).

SUMMARY AND CONCLUSIONS

1) Primary human breast cancers are rich in SSTRs.
2) Virtually all tumors in this series expressed multiple SSTR mRNAs.
3) SSTR5 is the predominant subtype, its mRNA being expressed in 90% of tumors, typically at very high levels.
4) Expression of SSTR3 and 4 mRNA also occurs in 70-90% of tumors. Expression of SSTR1 is somewhat less common.
5) There does not appear to be a correlation between the pattern of SSTR mRNA expression as assessed by RT-PCR analysis and tumor size, tumor grade, or ER/PR status. This may, however, change as our sample size increases. Two other potential problems may confound this type of analysis. One is the fact that receptor mRNA may not necessarily correlate with

functional SSTR protein. A second problem is that RT-PCR analysis of SSTR mRNA in tissue sections will not be able to distinguish expression of receptors in tumor cells and in surrounding normal peritumoral structures. These difficulties will be addressed in our subsequent tasks which will analyse SSTR expression at both mRNA and protein levels by *in situ* hybridization and immunocytochemistry in the subpopulations of tumor and surrounding normal cells.

6) Of the three SSTR subtypes 2, 3, 5 which are sensitive to OCT, SSTR5 and to a lesser extent SSTR2 and 3 are frequently expressed in breast cancer which now provides an adequate rationale for using OCT for tumor therapy in the NSABP and NCIC Clinical Trials that are about to begin.

(II) ***Expression of SSTR1-5 in Human Breast Cancer Tissue by Receptor Immunocytochemistry***

We succeeded in producing antipeptide rabbit polyclonal antibodies to all five human SSTRs. These antibodies have now been fully characterized for use in immunocytochemistry by the peroxidase method (Vectastain, ABC Kit) or immunofluorescence and confocal microscopy (Kumar et al, Endocrinology Rapid Communication, October 1997, in press). Figure 1 depicts confocal images of SSTR immunoreactivity in CHO-K1 cells stably transfected with hSSTR1-5. Cells expressing any one of the five SSTRs displayed positive fluorescence only when reacted with the corresponding SSTR primary antibody (panels A-E). There was no crossreactivity of any of the five SSTR antisera with another (nonhomologous) subtype. SSTR immunoreactivity in each instance was localized on the cell surface as well as intracellularly. The fluorescence was specific since it was not observed in nontransfected CHO-K1 cells (panels F-J), or when preimmune serum was substituted for SSTR primary antibody or when the primary antibody was absorbed with excess antigen. Using this panel of antibodies, we have begun an analysis of the expression of hSSTR1-5 in paraformaldehyde fixed sections of primary breast cancer samples. We have used both the immunoperoxidase method as well as immunofluorescence with rhodamine conjugated goat antirabbit IgG. Eleven samples (representing adjacent sections from 11 randomly selected tumors shown in Tables 1 and 2) have been fully analysed in our laboratory. The sections were then sent blinded to Dr. Peter Watson, a breast tumor pathologist and collaborator in Manitoba. Figure 2 shows the results of immunocytochemistry by the peroxidase method of a grade IV ductal NOS tumor stained with antibody to SSTR1-5. The brown reaction product signifies receptor immunoreactivity in clumps of tumor cells. There is strong expression of SSTR1, 2, 3, and 5. SSTR4 is relatively poorly expressed in only occasional cells. No specific staining was seen in control sections incubated with preimmune serum or antibody absorbed with excess peptide antigen. Expression of SSTR immunoreactivity was detected not only in tumor cells but also in peritumoral structures especially blood vessels and infiltrating immune cells. Figure 3 illustrates SSTR expression in another primary ductal NOS tumor (middle and right-hand panels) and in a peritumoral blood vessel (left-hand panels). There is weak expression of SSTR1 and SSTR2 in tumor cells surrounding the blood vessel. SSTR3 is present in the intima of the blood vessel as well as in tumor cells. SSTR4 is localized predominantly in the blood vessel being absent in the tumor cells. SSTR5 on the other hand is expressed strongly in the tumor cells but is absent in the blood vessel.

This study is ongoing but the results so far show a good correlation between SSTR protein expression as determined by immunocytochemistry with mRNA analysis by RT-PCR. The study, however, demonstrates a complex pattern of expression of SSTRs some of which preferentially occur in tumors, e.g. SSTR5 whereas others, e.g. SSTR3 and 4 which occur both in blood vessels and tumor cells. We are proceeding with our plans to analyse an additional 20 samples which should give us a sufficient number to be able to characterize the pattern of expression of the five subtypes in tumor and surrounding tissue and correlate this with our RT-PCR mRNA data and *in situ* hybridization data (to follow) to give a complete picture.

(III) ***SSTR Subtype Selectivity and Molecular Mechanisms Underlying PTP Regulation of Growth Inhibition and Induction of Apoptosis***

We have made considerable progress in each of the specific trasks assigned under this category. Our laboratory initially reported that SST induced apoptosis could be detected in phase synchronized proliferating but not growth arrested AtT-20 pituitary tumor cells. We have since established that in contrast to our findings in AtT-20 cells, such cytotoxic signalling can be monitored in exponentially growing MCF-7 breast cancer cells as well as in two other estrogen responsive human breast cancer cell lines, T47-D and ZR-75-1. In breast cancer cells, SSTR mediated antiproliferative signalling results in the induction of wild type p53, Bax and a pH-dependent, cation insensitive, endonuclease. We have shown that SST induced recruitment of cytosolic SHP1 to the cell membrane is an early event in its antiproliferative signalling. In parallel, we are characterizing SSTR subtype selective cytotoxic and cytostatic signalling in CHO-K1 cells individually expressing the five SSTR subtypes. Our results to date have provided new insights into the mechanism of hSSTR subtype selective antiproliferative actions and have established for the first time a novel G protein linked receptor mediated PTP-dependent signalling mechanism leading to apoptosis.

Salient features of our findings to date are:

1) OCT induces rapid apoptosis in MCF-7 breast cancer cells. This effect is potentiated by tamoxifen.
2) OCT does not activate membrane associated PTP directly in MCF-7 cells but rather induces translocation of cytosolic SHP1 to the membrane.
3) OCT induced apoptosis is signalled uniquely via hSSTR3 and does not require cell cycle arrest.
4) hSSTR3 induced apoptosis is associated with induction of wild type p53 and Bax.
5) SST induced cell death is characterized by DNA fragmentation caused by selective activation of Ca^{2+} , Mg^{2+} independent endonuclease.
6) SST elicits cytostatic action resulting in G_1 cell cycle arrest via other hSSTR subtypes (hSSTR5 > hSSTR2 > hSSTR4 > hSSTR1). This is associated with induction of the retinoblastoma protein pRB and p21 in a p53 independent manner.

These results are described more fully in the following publications:

1) Srikant, C.B. and Shen, S.H. Endocrinology 137:3461-3468, 1996. In this paper, we showed that hSSTR signalled antiproliferative action of MCF-7 human breast cancer cells is mediated by PTP. Such mediation does not involve activation of PTP by SST but rather recruitment of cytosolic PTP to the cell membrane. We have identified SHP1 as the principal cytosolic PTP recruited to the membrane by the action of SST.

2) Sharma, K., Patel, Y.C., and Srikant, C.B. Mol. Endocrinol. 10:1688-1697, 1996. This paper established for the first time that hSSTR mediated antiproliferative signalling leads to apoptotic cell death. Such cytotoxic signalling is transduced in a subtype selective manner uniquely by hSSTR3. Apoptosis triggered in this manner is associated with a dephosphorylation dependent induction of wild type p53 followed by induction of Bax and formation of oligonucleosomal DNA fragments. We showed using bivariate analysis of dual labelled cells by flow cytometry that hSSTR3 is signalled apoptosis does not involve cell cycle arrest, and that in dying cells wild type p53 and Bax are strongly induced. These findings provide the first demonstration of hormonal induction of p53 and apoptosis via a G protein coupled receptor in a subtype selective manner.

3) Sharma, K. and Srikant, C.B. Cell Growth and Differentiation 1997 (under review). In this study we demonstrate that OCT induced tumor cell apoptosis in MCF-7 cells is associated with induction of wild type p53 , bax, and a pH-dependent cation insensitive endonuclease. Induction of p53 preceded the onset of apoptosis and occurred in the absence of cell cycle arrest since markers of cell cycle arrest such as p21 and pRB were not induced in these cells.

4) Sharma, K. and Srikant, C.B. BBRC 1997 (submitted). In this study we have further characterized the events associated with hSSTR3 signalled apoptosis. We show that DNA degradation in CHO-K1 cells expressing hSSTR3 is due to selective activation of an endonuclease which is active under acidic pH conditions and does not require the presence of divalent cations such as Ca and Mg. Activation of this endonuclease parallels intracellular acidification in cells undergoing SST induced apoptosis.

5) Srikant, C.B., Cai, J., Sharma, K., and Shen, S.H. Proceedings of the Xth International Congress of Endocrinology, San Francisco, June 1996 (abstract). Inhibition of estrogen-stimulated growth of MCF-7 cells by OCT is potentiated by tamoxifen and involves recruitment of PTP-1C to the membrane. Here we show that potentiation of OCT induced antiproliferative signalling by tamoxifen is associated with increased recruitment of PTP-1C to the cell surface.

6) Sharma, K., Patel, Y.C., and Srikant, C.B. Proceedings of the 79th Annual Meeting of the US Endocrine Society, Minneapolis, June 1997 (abstract). We have compared the antiproliferative signalling via all five hSSTRs. In contrast to hSSTR3 which elicits a cytotoxic response, the other four subtypes transduce cytotoxic signals (hSSTR5 > hSSTR2 > hSSTR4 > hSSTR1). While hSSTR3 signalled apoptosis is dependent on the dose of the agonist over a concentration range 0.1-100 nM, the cytostatic signalling via the other subtypes

is seen only at concentrations > 10 nM. In hSSTR5 expressing cells, cytostatic signalling is associated with induction of pRB and p21 and leads to G_1 cell cycle arrest. A manuscript describing these findings is being prepared for submission.

CONCLUSIONS

1) Primary human breast cancers are rich in SSTRs.
2) All tumors analysed expressed multiple SSTR mRNAs.
3) SSTR5 is the predominant subtype, its mRNA being expressed in 90% of tumors, typically at very high levels.
4) Expression of SSTR3 and 4 mRNA also occurs in 70-90% of tumors, expression of SSTR1 is somewhat less common.
5) There does not appear to be a correlation between the pattern of SSTR mRNA expression as assessed by RT-PCR analysis and tumor size, tumor grade, or ER/PR status. This may, however, change as our sample size increases.
6) Of the three SSTR subtypes, 2, 3, 5 which are sensitive to OCT, SSTR5 and to a lesser extent SSTR2 and 3 are frequently expressed in breast cancer which now provides an adequate rationale for using OCT for tumor therapy in the NSABP-B29 and NCIC Clinical Trials that are about to begin.
7) OCT induces rapid apoptosis in MCF-7 cells, this effect is potentiated by tamoxifen.
8) OCT does not activate membrane associated PTP directly in MCF-7 cells but rather induces translocation of cytosolic SHP1 to the membrane.
9) OCT induced apoptosis is signalled uniquely via the SSTR3 subtype and does not require cell cycle arrest. This effect is associated with induction of wild type p53 and Bax.
10) SSTR3 induced cytotoxicity is characterized by DNA fragmentation caused by selective activation of Ca/Mg independent endonuclease. SST elicits cytostatic action resulting in G_1 cell cycle arrest via SSTR subtypes 1, 2, 4, 5 (SSTR5 > SSTR2 > SSTR4 > SSTR1). This is associated with induction of the retinoblastoma protein pRB and p21 in a p53 independent manner.

TABLE 1

EXPRESSION AND RELATIVE ABUNDANCE OF mRNA FOR SSTR1-5 IN PRIMARY DUCTAL NOS BREAST CANCER SAMPLES IN RELATION TO TUMOR HISTOLOGY AND ER/PR STATUS

Samp#	Type	GR	INV%	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	ER	PR	#LN+	TS
1	Ductal NOS	8	30	6	1	0	1.3	8.8	1	11	N	99.8
2	Ductal NOS	6	60	10	9	0.2	0	8	146	216		1.4
4	Ductal NOS	8	50	0	4	2.6	0.2	0	0	9.1	P	
5	Ductal NOS	4	70	7.3	7	12	0.5	10	232	22	N	3.7
6	Ductal NOS	9	60	0	3.2	0	0.4	10	0.7	5.4	P	2.0
7	Ductal NOS	6	30	2	5.4	1	9.8	17	140	101		99.8
8	Ductal NOS	9	70	0	0	ND	13	0	144	377	N	1.5
9	Ductal NOS	4	40	10	8	0.2	9.1	17	78	44		
10	Ductal NOS	8	40	0	3	0	0.1	0	1.4	6.1	P	
12	Ductal NOS	8	50	0	0	4	2.1	0	1.4	5.5	N	4.0
14	Ductal NOS	7	40	0	3.4	0	1.4	0	1.2	6.7	P	3.2
15	Ductal NOS	5	60	0	4	0	1.9	6	247	56	P	4.7
18	Ductal NOS	8	25	9	0	0	2	9	4.4	1.5	N	6.1
19	Ductal NOS	9	50	0	26	2.8	0.3	24	1.5	4.7	N	2.5
20	Ductal NOS	5	30	0	5	0	1.6	18	10.4	101	N	2.0
21	Ductal NOS	9	80	11	4	8.8	9.6	10	5.5	11.5	P	3.0
22	Ductal NOS	7	30	0	4	0	1	18	11.8	15.8	N	3.2
23	Ductal NOS	8	50	6	8	13	3.1	14	2.1	8.1	N	99.8
25	Ductal NOS	7	70	10	6.2	11	12	14	18.7	4.4		
26	Ductal NOS	7	30	5	0	0	6.1	6.7	43	61	N	99.8
27	Ductal NOS	6	40	5	0	9	3.4	10	60	288	N	
28	Ductal NOS	6	30	0	0.4	0	8.4	12.5	60	197		3.5
29	Ductal NOS	4	50	0	4	0	9.3	7.5	225	144	P	2.6
30	Ductal NOS	5	25	0	0	9	6.7	14	50	55	N	4.0
32	Ductal NOS	7	50	0	0	0	3.4	7.5	72	38		5.0
34	Ductal NOS	7	40	0	0	0	3.4	7.5	96	24		
35	Ductal NOS	9	90	0	0	8.8	3.5	0	46	6.6		2.6
37	Ductal NOS	9	55	11	0	8.7	5.5	0	0.3	0	P	8.0
38	Ductal NOS	8	55	0	0	8.8	2.3	0	1.6	4.2		
39	Ductal NOS	5	40	ND	0	8.4	8	0	110	5.7	N	2.6

40	Ductal NOS	4	30	11	0	11	2.5	7.5	113	20		
42	Ductal NOS	6	45	0	0	9.2	10	8.2	97	96	P	7.5
43	Ductal NOS	6	40	5.5	0	6.6	9.8	9.3	4.9	84	P	3.5
44	Ductal NOS	7	50	6.4	0	6.8	8.2	12	28	20	P	1.4
46	Ductal NOS	9	40	21.1	0	16	2.9	4.3	0	12		
47	Ductal NOS	9	50	54	0	8.6	6.3	7.6	0.6	0	N	6.0
49	Ductal NOS	6	40	0	0	2	1.5	4.8	117	273	P	
50	Ductal NOS	8	40	0	0	2.2	3.3	5.5	74	98	N	2.5
51	Ductal NOS	6	30	17	0	19	0	0	1.5	6	P	99.8
52	Ductal NOS	9	50	10	0	8	2.5	3.3	0	8	P	2.5
54	Ductal NOS	6	30	1.3	6.3	4.1	9	15	104	10.7		
55	Ductal NOS	8	30	3.1	6	7.1	8	12	13.7	14.6		
56	Ductal NOS	9	70	4	5	6	6.1	13	93	141		
57	Ductal NOS	9	40	6.1	22	1.7	7	17	3.8	4.1		
58	Ductal NOS	5	20	3.2	4.5	5	ND	32	39	42		
59	Ductal NOS	5	20	4	5.7	6	0	15	94	136		
60	Ductal NOS	7	40	4	6.8	7	0	9	60	15.3		
61	Ductal NOS	7	45	4.3	4.7	6.3	4.7	9	71	69		
62	Ductal NOS	6	30	3.2	4.8	5.1	3.9	20	16.9	305		
63	Ductal NOS	5	25	14.1	6.7	4.7	5.9	6.8	17.3	42		
64	Ductal NOS	7	30	6	7.7	7	4.4	8.5	216	274		
66	Ductal NOS	7	25	5.3	6.5	8.2	4	12	201	16.3		
67	Ductal NOS	6	30	5	5.8	7.7	3.5	7.8	13.6	26		
68	Ductal NOS	7	60	5.5	5.4	6.2	0	9	111	13.9		
69	Ductal NOS	6	25	6.2	5.4	5.2	5.1	1	37	8		
70	Ductal NOS	6	25	ND	ND	1.8	5.3	12	55	48		
71	Ductal NOS	9	30	7	8.7	7	0	8.5	5.2	8.8		
72	Ductal NOS	8	30	9	3.7	5.6	11	11	4.9	9.4		
73	Ductal NOS	5	30	7.2	5.6	5.7	4	9	85	138		
74	Ductal NOS	9	50	8.2	1.5	7.9	2.5	6.4	2.1	5.8		
75	Ductal NOS	8	20	ND	0	3.7	0	7	172	241		
76	Ductal NOS	5	20	0	6.5	7.8	5	1.5	11	177		
79	Ductal NOS	8	50	1.8	12	6.9	6	5.4	15.1	15.6		
82	Ductal NOS	9	75	ND	6	7.9	0	ND	0.2	4.3		
83	Ductal NOS	7	50	0	0.7	6	4.7	6.7	1.9	7.8		
84	Ductal NOS	9	50	3.2	5.5	5.4	4	6.2	1	1.3		

85	Ductal NOS	8	50	6	7	1.8	4.2	12	32	11.1		
87	Ductal NOS	7	75	10	10	5	0	7	0.3	4.7		
88	Ductal NOS	7	50	0	3	3.2	ND	7.6	85	28		
89	Ductal NOS	9	60	0	ND	3.7	ND	5.5	1.4	6.3		
90	Ductal NOS	7	50	4.2	5	7.4	2.5	8.1	1.5	8.3		
91	Ductal NOS	7	75	0	0	13	0	11	31	2.9		
92	Ductal NOS	9	25	0	0	0	5	6.9	1.5	6.3		
93	Ductal NOS	9	40	5.3	0	4.5	ND	8.8	1.2	3.4		
95	Ductal NOS	5	50	6.5	5.4	1.1	4.5	9.7	23	18.1		
96	Ductal NOS	8	30	4.1	2.3	0.13	4.1	ND	0.6	0.5		
97	Ductal NOS	5	40	4	6	3.4	7.4	7	36	157		
98	Ductal NOS	8	50	0	0	7.5	ND	4.1	34	19.7		
99	Ductal NOS	5	75	0	10	8.4	3.3	4.7	57	17.9		
100	Ductal NOS	5	75	0	11	6.4	6.2	3.7	143	44		
101	Ductal NOS	5	40	6.4	9	3.4	6.3	4.3	143	44		
102	Ductal NOS	5	30	0	3.3	7.9	4.6	5.4	48	5.5		
103	Ductal NOS	4	90	0	7	13	6	6.8	13.3	23		
104	Ductal NOS	5	75	0	10	8.5	6.6	7.6	108	14.1		
105	Ductal NOS	5	75	0	14	7.5	5.1	7.2	120	30		
106	Ductal NOS	5	50	0	13	8.4	5	6.8	249	177		
107	Ductal NOS	6	75	1.1	12	7.6	7	4.7	74	63		
108	Ductal NOS	6	50	6.2	11	3.7	5.3	3.3	10	14.4		
109	Ductal NOS	8	60	3	13	7.2	7.1	4.9	2.8	4.5		
110				7.3	7	3	5	8.2				
111				4.4	0	2.7	6.1	10				

SSTR1-5 mRNA (expressed in densitometric units) as fold increase compared to actin mRNA.
GR = tumor grade (Nottingham scale)
INV = % invasiveness
#LN+ = lymph node status (P, positive; N, negative)
ER = estrogen receptor
PR = progesterone receptor
TS = tumor size

TABLE 2†

EXPRESSION AND RELATIVE ABUNDANCE OF mRNA FOR SSTR1-5 IN TUBULAR/LOBULAR/MIXED PRIMARY BREAST CANCER SAMPLES COMPARED WITH TUMOR HISTOLOGY AND ER/PR STATUS

Samp#	Type	GR	INV%	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	ER	PR	#LN+	TS
3	Tubular	3	30	0	4	0	0.4	12	128	541		1.7
11	Tubular mix	4	50	10	8	0.7	1.4	29	2.6	25	N	1.0
13	Tubular mix	4	30	0	3	0.3	9.1	18	4.2	9.6		99.8
16	Lobular	5	50	0	5.4	0	1.2	6.7	1.5	15.3	N	6.0
17	Tubular	4	30	0	6.4	0	2	16	67	24	N	2.2
24	Duct-Lob mix	6	25	6	5	0.5	10	17	16.3	53	N	
33	Duct-Lob mix	6	35	0	0	0	0	4.4	19.8	81	N	
41	Tubular	4	30	0	0	8.9	2.5	0	105	35		3.0
45	Inv lobular	5	30	0	0	4	4.7	0	24	59	N	3.2
48	Tubular mix	4	40	21	0	16	2.9	4.3	85	125	N	1.2

† abbreviations as in Table 1

TABLE 3

MEAN LEVEL AND INCIDENCE OF mRNA FOR EACH INDIVIDUAL SSTR SUBTYPE IN THE GROUP OF DUCTAL NOS PRIMARY BREAST CANCERS SHOWN IN TABLE 1

	SSTR1	**SSTR2**	**SSTR3**	**SSTR4**	**SSTR5**
No analysed	87	89	90	86	89
No positive	54	62	77	76	80
% positive	62%	70%	86%	88%	90%
mRNA level (mean $\pm$ SE)	4.6 ± 0.74	4.75 ± 0.52	5.54 ± 0.43	4.73 ± 0.34	8.3 ± 0.58

TABLE 4

MEAN LEVEL AND INCIDENCE OF mRNA FOR EACH INDIVIDUAL SSTR SUBTYPE IN THE GROUP OF TUBULAR/LOBULAR/MIXED PRIMARY BREAST CANCERS SHOWN IN TABLE 2

	SSTR1	**SSTR2**	**SSTR3**	**SSTR4**	**SSTR5**
No analysed	10	10	10	10	10
No positive	3	6	6	9	8
% positive	30%	60%	60%	90%	80%
mRNA level (mean $\pm$ SE)	3.7 ± 2.2	3.2 ± 0.96	3 ± 1.7	3.4 ± 1.1	10.7 ± 2.95

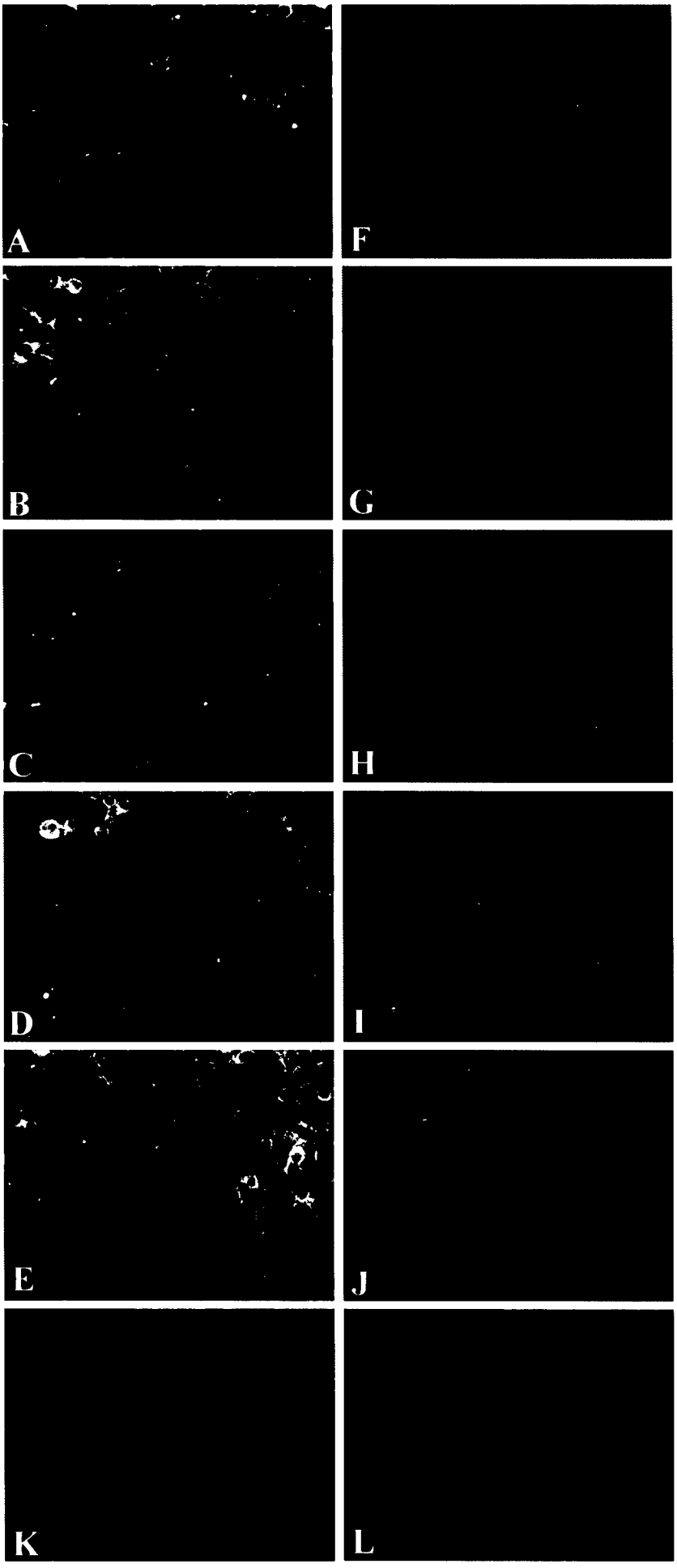

Figure 1

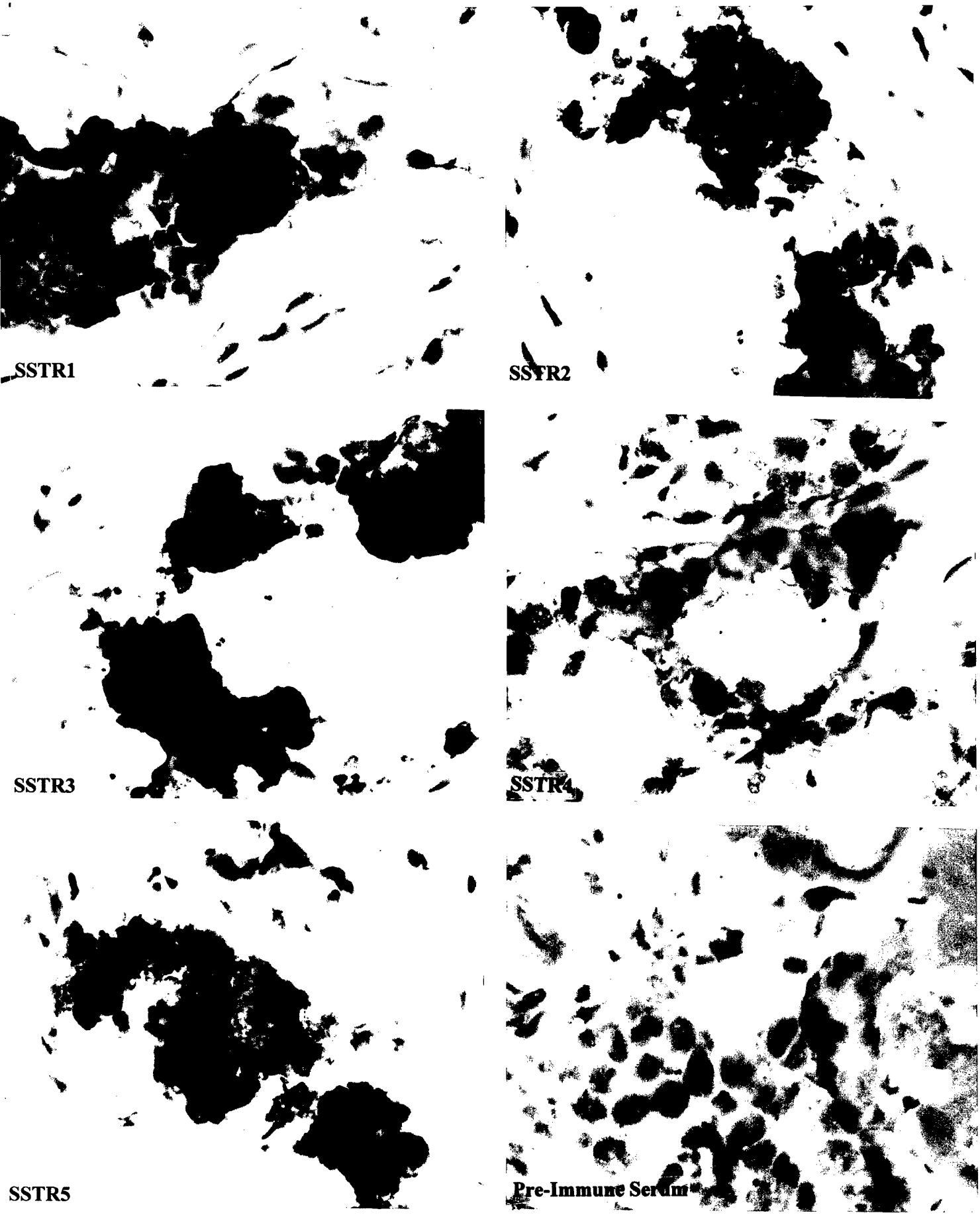

Figure 2

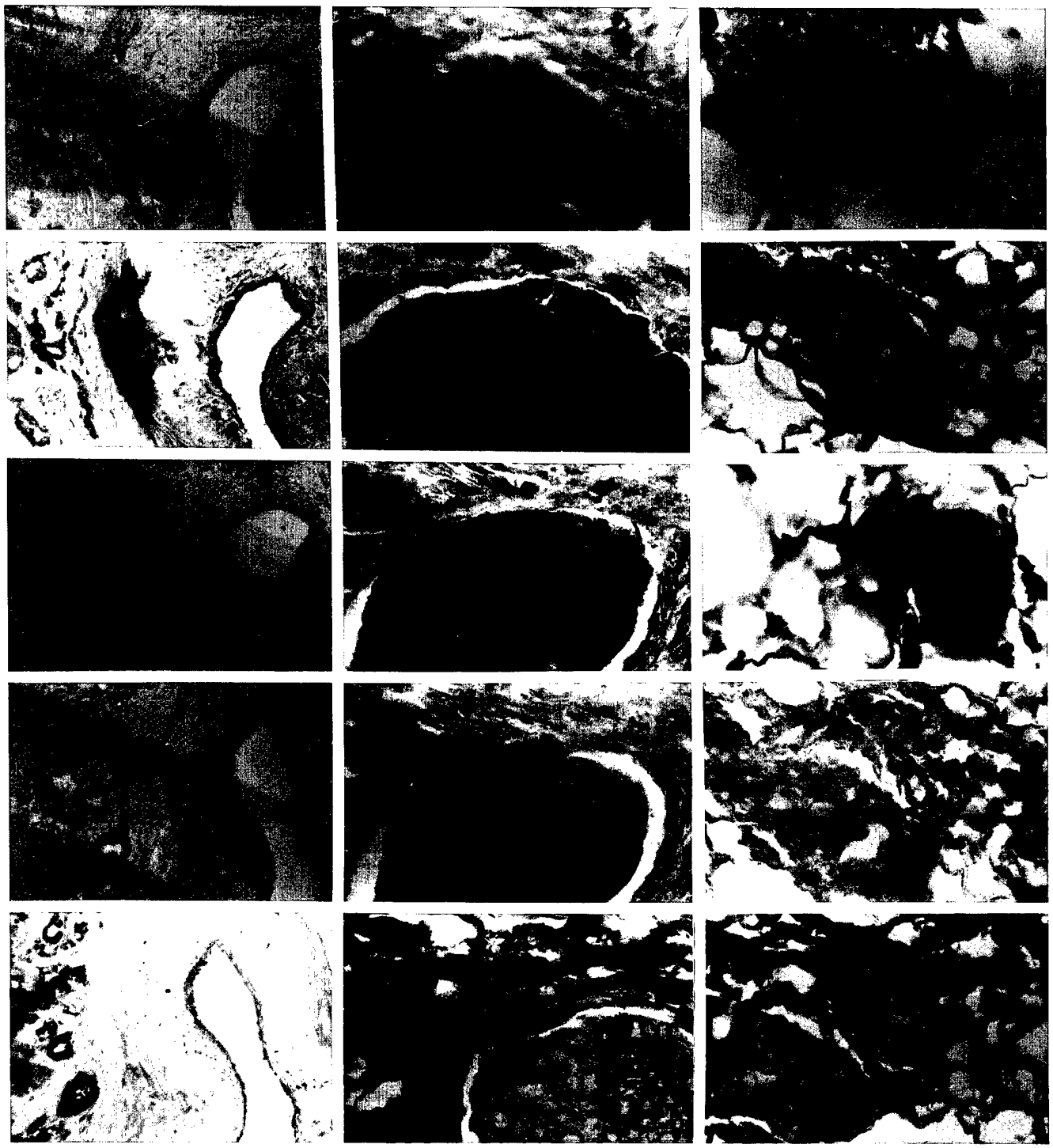

Figure 3

FIGURE LEGENDS

FIGURE 1:

Confocal images depicting SSTR immunofluorescence in CHO-K1 cells stably expressing hSSTR1-5. A - SSTR1, B - SSTR2, C - SSTR3, D - SSTR4, E - SSTR5. The corresponding nontransfected CHO-K1 cells (F-J) are devoid of immunoreactivity. K = 3 immune serum control, L = control without addition of primary antiserum. Scale bar = 50 μM.

FIGURE 2:

Adjacent sections from a grade IV ductal NOS primary cancer sample analysed by peroxidase immunohistochemistry for SSTR1-5 antigens. This tumor was positive for all five SSTR mRNAs by RT-PCR. Compared to a typical preimmune serum control shown in the lower right panel, there is strong expression of immunoreactivity for SSTR1, 2, 3, and 5 in many tumor cells and of SSTR4 in occasional cells.

FIGURE 3:

Expression of SSTR1-5 in two separate sections from a ductal NOS tumor (middle and right-hand panels) as well as in a peritumoral blood vessel (left-hand panel). Note the rich expression of SSTR3 antigen in mammary epithelial cells. By contrast, SSTR5 in this tumor is predominantly expressed in peritumoral structures with minimal expression of the receptor in tumor cells. In the case of blood vessels (left-hand panels) SSTR3 is present in the intima as well as in tumor cells. SSTR4 is localized predominantly in the blood vessel being absent in the adjacent tumor cells. SSTR5 on the other hand is expressed strongly in the tumor cells but is absent in the blood vessels.

APPENDIX

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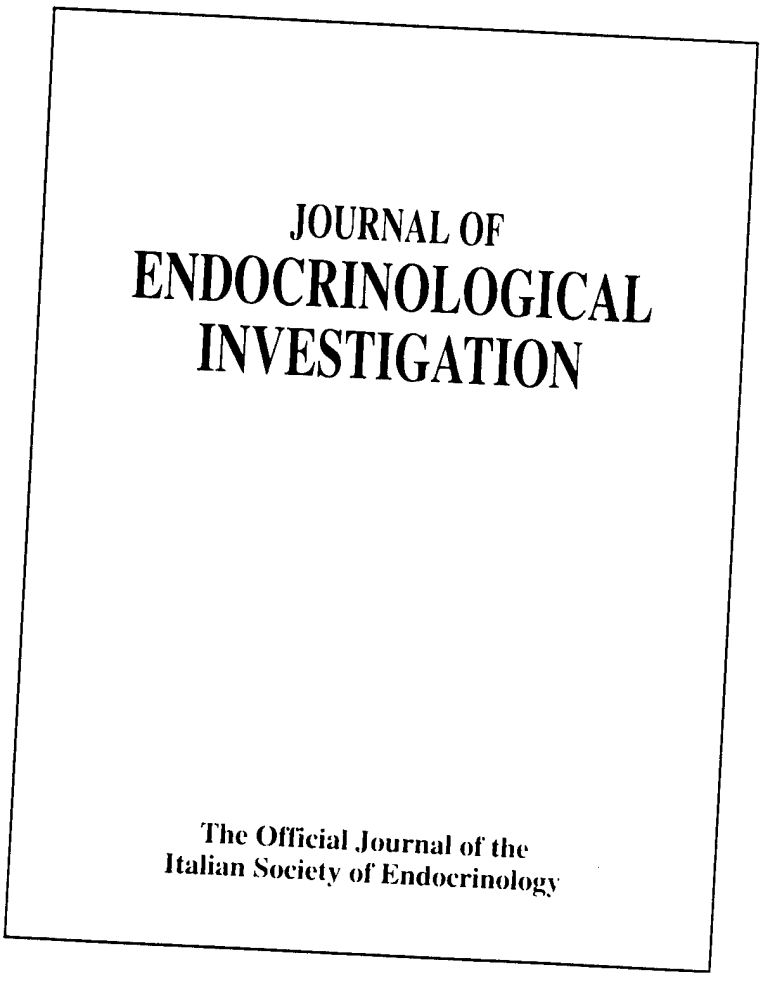

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REVIEW ARTICLE

Molecular pharmacology of somatostatin receptor subtypes

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INTRODUCTION

Somatostatin (SST) constitutes a multi gene peptide family with two principal bioactive products, SST-14 and SST-28 (1-4). In vertebrates these two peptides have evolved from separate SST-14 and SST-28 encoding genes in fish to a single mammalian SST gene encoding a common precursor which is differentially processed to generate tissue-specific amounts of SST-14 and SST- 28 (3-5). The SST gene is expressed in neurons and endocrine-like cells found throughout the central and peripheral nervous systems, in the endocrine pancreas, gut, thyroid, kidneys, adrenals, reproductive organs, and immune cells (3, 6, 7). Recently, a second SST-related gene termed cortistatin (CST) has been described in humans and rat which gives rise to two putative cleavage products comparable to SST-14 and SST-28 (8, 9). These consist of human CST-17 and its rat analog CST-14, and human and rat CST-29. Unlike the broad distribution of SST, gene expression of CST is restricted to the cerebral cortex (8). Secretion of SST is stimulated by ions, nutrients, other neuropeptides, various neurotransmitters, hormones, growth factors, and cytokines (3, 4, 6).

Steady state SST mRNA levels are stimulated by GH, IL-1, TNFα, glucocorticoids, testosterone, estradiol, and NMDA receptor agonists and inhibited by glucocorticoids and insulin (3, 4, 6). Among the intracellular mediators known to modulate SST function are Ca^{2+}, cAMP, cGMP, and activators of protein kinase C (3, 4, 6, 10-13). Somatostatin acts on multiple targets including the brain, gut, pituitary, endocrine and exocrine pancreas, adrenals, thyroids, kidneys, and immune cells (3, 6, 14, 15). Its action includes inhibition of virtually every known endocrine and exocrine secretion, motor, sensory, behavioural, cognitive, and autonomic effects, as well as effects on intestinal motility, vascular contractility, cell proliferation, and on intestinal absorption of nutrients and ions (3, 6, 14, 15). Synthetic rat CST-14 shares 11 of 14 residues with SST-14 including the biologically active core, but nonetheless, exerts effects identical to as well as distinctive from those of SST-14 (8). The pleiotropic effects of SST can be resolved into 3 cellular processes that are modulated by SST: neurotransmission, secretion and cell proliferation. Today SST is best regarded as a major biological system which regulates a variety of physiological functions both locally as a neurotransmitter, neuromodulator, or paracrine/autocrine regulator and systemically as a true hormone. In the nervous system, the peptide is a physiological regulator of neocortical, striatal, limbic, and hypothalamic neurons (3, 6, 16). In peripheral tissues, it is important in endocrine, gastrointestinal, and immune cell functions (3, 6, 7). Compelling evidence has implicated SST in the pathophysiology of several diseases including neoplasia, neurodegenerative disease, AIDS, and diabetes (3, 4, 6, 16-18). This review focuses on recent progress in the molecular biology and pharmacology of SST receptors (SSTRs). For additional coverage of this rapidly expanding topic, the reader is referred to several earlier reviews (14, 15, 17, 19-22).

THE SOMATOSTATIN RECEPTOR FAMILY

The diverse effects of SST are mediated via high affinty plasma membrane SSTRs which have been demonstrated by binding analysis using whole cell or plasma membrane fractions, *in vivo* and *in vitro* autoradiography, covalent crosslinking, and purification of the solubilized SSTR (23-32). With these methods, SSTRs have been shown to occur in varying densities in brain, gut, pituitary, endocrine and

Key-words: Somatostatin, somatostatin receptor, SSTR subtypes.

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exocrine pancreas, adrenals, thyroid, kidneys, and immune cells. SSTRs have also been localized in hamster insulinoma and Rin M5f islet tumor cells (32, 33), GH_3/GH_4C_1, and AtT-20 pituitary tumor cells (23, 27, 28), AR42J and Mia Paca2 pancreatic tumor cells (34, 35), and in human breast cancer (36), neuroblastoma (37), glioma (38), and leukemic and myeloma cell lines (39). Photoaffinity labelling and purification studies have provided evidence for the existence of several different molecular species of SSTR proteins which are expressed in a tissue-specific manner and which exhibit selective agonism for SST-14 or SST-28 (28, 29). Beginning in 1992, the structure of SSTRs was elucidated by molecular cloning (14, 15, 40-51). Five individual subtypes were rapidly identified and shown to consist of a family of heptahelical G protein coupled receptors (GPCR) (14, 15, 40-51). Yamada et al. in Bell's laboratory cloned the first two SSTRs (SSTRI and SSTR2) from human islet RNA (40). SSTR3,4,5 were subsequently cloned from human, rodent, bovine, and porcine tissues using a variety of different techniques (14, 15). Our laboratory has focussed on the human genes and accordingly the human SSTR subtypes will be emphasized throughout this review (14, 17). Human SSTRs (hSSTRs) are encoded by a family of 5 non-allelic genes localized on separate chromosomes (Fig. 1, Table 1). Four of the genes are intronless, the exception being SSTR2 which gives rise to

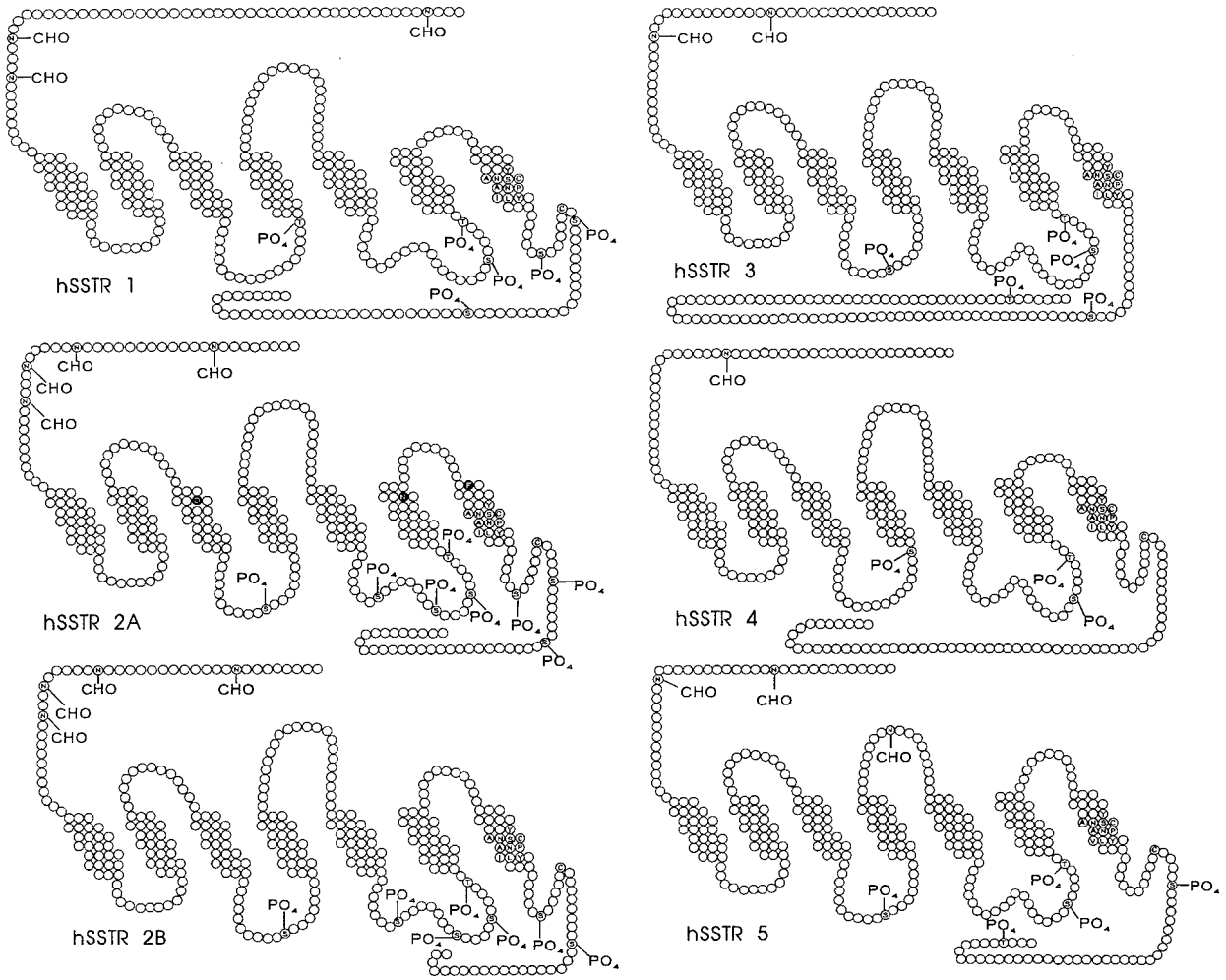

Fig. 1 - *Schematic depiction of the 7 transmembrane topology of the 6 putative hSSTR proteins. The conserved YANSCANPI/VLY sequence in the 7th TM of each receptor is shown as is the Cys palmitoylation membrane anchor in the C-tail of hSSTR1, 2, 4, 5. CHO-potential sites for N-linked glycosylation; PO_4 potential sites for phosphorylation by protein kinase A, protein kinase C and casein kinase. Residues ASP^{122}, Asn^{276}, and Phe^{294} in TMs III, VI, and VII respectively of SSTR2A which have been proposed to form part of a ligand binding pocket for SMS201-995 ligand (ref. 73) are shown by the closed circles.*

Table 1 - *Characteristics of the five cloned subtypes of human somatostatin receptors*†.

	SSTR1	SSTR2A	SSTR2B	SSTR3	SSTR4	SSTR5
Chromosomal localization	14q13	17q24	17q24	22q13.1	20p11.2	16p13.3
Amino acids	391	369	356	418	388	363
m.w. (kDa)	42.6	41.3	39.9	45.7	41.9	39.1
mRNA (kb)	4.8	8.5 (?)	2.5 (?)	5.0	4.0	4.0
G protein coupling	+	+	NT	+	+	+

† from refs. 14, 15; NT, not tested

spliced variants SSTR2A and SSTR2B, which differ only in the length of the cytoplasmic C-tail (52, 53). There are thus six putative SSTR subtypes of closely related size, each displaying a 7 transmembrane domain (TM) topology (Fig. 1). The six hSSTRs display single or multiple sites for N-liked glycosylation within the amino terminal segment and second extracellular loop (ECL) and putative phosphorylation sites located intracellularly within the second and third intracellular loops and in the cytoplasmic C-terminal segment (Fig. 1). hSSTR 1, 2, 4, 5 display a conserved cysteine residue 12 amino acids downstream from the 7th TM which may be the site of a potential palmitoyl membrane anchor. All SSTR isoforms that have been cloned so far from humans as well as other species possess a highly conserved sequence motif YANSCANPI/VLY in the 7th TMD which serves as a signature sequence for this receptor family.

Overall, there is 39-57% sequence identity among the various members of this family with SSTR1 and SSTR4 showing the highest sequence identity (14, 15). The individual subtypes display a remarkable degree of structural conservation across species. For instance, there is 94-99% sequence identity between the human, rat (r) and mouse (m) isoforms of SSTR1, 93-96% sequence identity between human, rat, mouse, porcine, and bovine isoforms of SSTR2, and 88% sequence identity between the rat and human isoforms of hSSTR4 (14, 15). SSTR3 and SSTR5 are somewhat less conserved showing 82-83% sequence identity between the human and rodent homologues (14, 15). The nearest relatives of the SSTRs are the opioid receptors whose δ subtype displays 37% sequence similarity to the mSSTR1 (54). A putative heptahelical receptor protein with ~ 20% sequence similarity with SSTR3 has been described on chromosome 3 of *c. elegans* and may represent a primodial SSTR (55). Several novel genes with sequence similarity to the SSTR family have recently been described. Two of these (SLC-1/GPR-24, GPR-25) display 34-40% sequence homology with the membrane spanning domains of SSTRs but do not contain the YANSCANPILI/VY motif (56, 57). These receptors do not bind SST or any other known ligand and therefore, currently remain as orphan receptors which probably belong to a related family.

AGONIST BINDING

Over the years, many different SST peptide analogs have been synthesized for investigational and clinical use (Fig. 2) (58-61). Structure-function studies

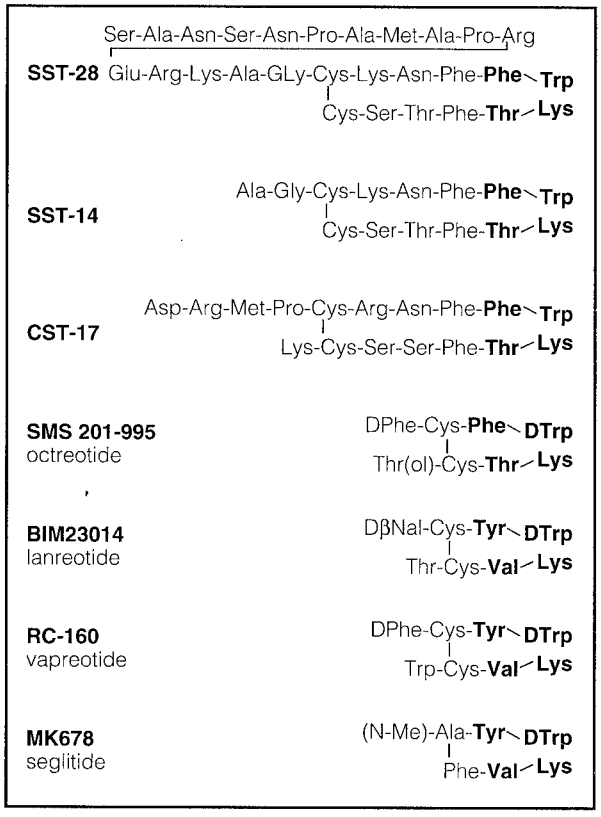

Fig. 2 - *SST-Receptor Agonists.*

of SST-14 have shown that amino acid residues Phe^7, Trp^8, Lys^9 and Thr^{10} which comprise a β-turn are necessary for biological activity with residues Trp^8 and Lys^9 being essential whereas Phe^7 and Thr^{10} can undergo minor substitutions, e.g. Phe → Tyr and Thr → Ser or Val. The general strategy for designing SST analogs has been to retain the crucial Phe^7, Trp^8, Lys^9, Thr^{10} segment and to incorporate a variety of cyclic and bicyclic restraints to stabilize the β-turn around the conserved residues. In this way, a number of octapeptide and hexapeptide molecules have been designed which show greater metabolic stability and some pharmacological selectivity compared with SST-14. All five SSTR subtypes bind SST-14 and SST28 with high affinity (Table 2). SSTRI-4 display weak selectivity for SST-14 whereas SSTR5 exhibits 10-15 fold higher affinity for SST-28 than SST-14 and appears to be SST-28 selective (14, 62-64). It should be noted that all five SSTRs bind SST-14 with comparable (nM) affinity and that the subtype selectivity of SST-28 for hSSTR5 is relative rather than absolute since the other four SSTRs are also capable of high affinity binding to this ligand. SST-28 binds rSSTR5 > hSSTR5 (50, 51). This property as well as the ability of other SST analogs to bind differently to rat and human SSTR5 may be explained by the relatively greater sequence divergence between these two receptor homologs compared to the other SSTR subtypes. Synthetic replicates of the putative CST products hCST-17 and rCST-14 bind all five hSSTRs with nanomolar affinity (Table 2) (8, 9). Synthetic rCST-29 binds to hSSTR1, 3,5 with comparable affinity to SST-14. Its affinity for SSTR2 and SSTR5 is 5-100 fold lower than that of SST-14 and SST-28. The octapeptide analogs, SMS201995 (SMS, Octreotide), BIM23014 (Lanreotide), and RC160 (Vapreotide), that are in clinical use, as well as the hexapeptide MK678 (Seglitide) bind to only three of the five hSSTR subtypes displaying high affinity for types 2 and 5 and moderate affinity for type 3 (with the exception of SMS) (62-64). SMS in our hands binds well to hSSTR3 (K_i 4.4 nM) whereas others have reported only modest binding to this subtype (IC_{50} ~ 35 nM) (62, 64). The binding affinity of SMS, BIM23014, RC160, and MK678 for subtypes 2 and 5 is comparable to that of SST-14 indicating that they are neither selective for these subtypes nor more potent than the endogenous ligands. Based on structural similarity and reactivity for octapeptide and hexapeptide SST analogs, the receptor family can be divided into two subclasses, SSTR2,3,5 which react with these analogs and constitute members of one subgroup, and SSTR1,4

Table 2 - *Agonist selectivity of cloned human somatostatin receptors.*

AGONIST	K_i (nM)					
	SSTR1	SSTR2A	SSTR3	SSTR4	SSTR5	REF.
SST-14	1.1	1.3	1.6	0.53	0.9	62
SST-28	2.2	4.1	6.1	1.1	0.07	62
hCST-17†	7	0.6	0.6	0.5	0.4	9
rCST-29	2.8	7.1	0.2	3	13.7	*
SMS 201-995	>1000	2.1	4.4	>1000	5.6	62
BIM 23014	>1000	1.8	43	66	0.62	62
RC-160	>1000	5.4	31	45	0.7	62
MK 678	>1000	1.5	27	127	2	62
NC 8-12	>1000	0.024	0.09	>1000	>1000	62
BIM 23197†	>1000	0.19	26.8	>1000	9.8	64
BIM 23268†	18.4	15.1	61.6	16.3	0.37	64
des AA^{1,2,5} [DTrp^8 IAmp^9] SST-14	3.2	>1000	>1000	4.3	>1000	*

rCST-29	Gln-Glu-Arg-Pro-Leu-Gln-Gln-Pro-Pro-His-Arg-Asp-Lys-Lys-Pro c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]Lys
NC8-12	D-Phe-c [Cys-Tyr-D-Trp-Lys-Abu-Cys] Nal-NH_2
BIM23 197	Hepes-D-Phe-c [Cys-Tyr-D-Trp-Lys-Abu-Cys] -Thr-NH_2
BIM23268	c[Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys]-NH_2

Abu, aminobutyric acid; Nal, β-(2-naphthyl)alanine; IAmp, N-p-isopropyl-4-aminomethyl-Lphenylalanine.
*Data of Hukovic, N. and Patel, Y.C., unpublished; † binding potencies shown as IC_{50}.

which react poorly with these compounds and fall into another subgroup. Other than SST-14 and SST-28 and some of their structural derivatives, there are no current SST compounds capable of binding to all five subtypes. Investigation of a wide range of other SST analogs for subtype selectivity has identified compounds, e.g. NC8-12 with modest 20-50 fold selectivity for hSSTR2 and 3 (62), DC23-60, EC5-21, and BIM23197 with ~19-50 fold selectivity for hSSTR2 (62, 64), and BIM23268 with ~ 45 fold selectivity for hSSTR5 (64). The analog Des-$AA_{1,2,5}$ [D-Trp^8 IAMP^9] SST-14 has been recently described as a SSTRI selective compound (65). In our hands, however, this peptide also binds to hSSTR4 and appears to be a prototypic agonist for the SSTRI, 4 subclass (Table 2). Very recently, the first potential SSTR peptide antagonists have been designed using the SMS skeleton as template (66). One such compound [AC-4-NO_2 - Phe-c (D-Cys-Tyr-DTrp-Lys-Thr-Cys) D-Trp-NH_2] binds to hSSTR2 and 5 with nM affinity but produces antagonism by blocking receptor-effector coupling to adenylyl cyclase (66).

LIGAND BINDING DOMAIN

Because of the difficulty in crystalizing GPCRs, attempts to elucidate the structural and functional domains of these molecules have resorted to indirect methods such as site-directed mutagenesis and receptor chimeras. Based on such studies, the ligand binding site of GPCRs has been postulated to consist of a number of noncontiguous amino acid residues which form a binding pocket within the folded receptor structure (67, 68). An alternative model, however, proposes that there is no binding cavity as such, but that agonists interact with crucial residues in the extracellular and/or transmembrane domains to stabilize preformed, active receptor conformations (69). Whereas large protein ligands such as glycoprotein hormones interact principally with the amino terminal segment, and biogenic amines bind to residues in the TMs, the ligand binding site of peptide agonists comparable to SST typically involves residues in the ECLs or both the ECLs and the TMs (69-72). By exploiting the differential ability of SMS to bind to hSSTR2 but not to hSSTR1, Kaupman et al. systematically mutated hSSTR1 to resemble hSSTR2 (73). They found two crucial residues, Gln_{291} and Ser_{305}, in TMs VI and VII respectively of hSSTRI, substitution of which to the corresponding residues Asn^{276} and Phe^{294} in hSSTR2 increased the affinity of hSSTRI for SMS and other octapeptide analogs 1000 fold (Fig. 1). By molecular modelling using these identified residues, as well as the known structure of SMS, Kaupman et al. have postulated a binding cavity for SMS involving hydrophobic and charged residues located exclusively within TMs III-VII (73). Their findings predict that the core residues Phe^7, Trp^8, Lys^9, Thr^{10} of SMS interact with Asn^{276} and Phe^{294} located at the outer end of TMs VI and VII respectively (present in SSTR2 but not in SSTRI) which provide a hydrophobic environment for lipophilic interactions with Phe^7, Trp^8, Thr^{10}, and Asp^{137} in TM III which anchors the ligand by an electrostatic interaction with Lys^9 (73) (Fig. 1). SMS binds poorly to hSSTRI because of the presence of residues Gln^{291} and Ser^{305} located close to the extracellular rims of TM helices VI and VII which prevent the short peptide from reaching deep within the pocket, whereas the corresponding residues Asn^{276} and Phe^{294} in SSTR2 provide for a stable interaction with the disulphide bridge of SMS. Because of their greater length and flexibility, the natural ligands SST-14 and SST-28, can presumably adopt a conformation that allows their entry into the binding pocket of all five SSTRs. Such a model is consistent with mutational studies of the Asp residue in TM III which abolishes ligand binding although it is not known whether this is due to a direct interaction of the residue with SST ligands or to a secondary alteration in receptor structure (74, 75). The results of Fitz-Patrick and Vandlen are also consistent with the notion that short SST analogs bind to residues in TMDs VI and VII. Using chimeric mouse SSTR1/SSTR2 receptors, these workers demonstrated that swapping the ECL III and upper segments of the adjacent TMs VI and VII of SSTR2 (encompassing the critical Asn and Phe residues) with the comparable domains of SSTR1 resulted in a loss of affinity for the hexapeptide MK678 (76). It should be noted that of all the residues identified by mutagenesis as being important in recognizing SMS and MK678, none have been shown directly to be critical for binding the natural ligands SST14 and SST-28. Furthermore, the assumption by Kaupman et al. that SSTR2 shares a common ligand binding pocket with the other SSTRs may not be generally applicable since closely related GPCRs may feature different sets of epitopes for binding of a common ligand. The involvement of the extracellular domains for binding SST ligands has been investigated by Greenwood et al. using amino terminal deletion mutants or conservative segment exchange mutagenesis for the three ECLs of hSSTR5 (77). Their results predict a potential contribution of ECL2 (but not of ECL1, ECL3 or the amino terminal segment) to binding of the natural SST ligands (SST-14, SST-28) as well as SMS. The overall

model that emerges from these studies suggests a binding domain for SST ligands made up of residues within TMs III-VII with a potential contribution by ECL2, and is consistent with other peptide binding GPCRs, e.g. NK1, AT2, GnRH, which interact with residues in both ECLs and TMs (69-72).

G PROTEIN COUPLING AND SIGNAL TRANSDUCTION

Coupling of Endogenous SSTRs

Ligand activation of endogenous SSTRs is associated with a reduction in intracellular cAMP and Ca^{2+} and stimulation of protein phosphatases due to receptor activation of four major membrane signalling pathways each involving a pertussis toxin sensitive GTP binding protein (Fig. 3) (14, 15, 21, 22): (1) receptor coupling to adenylyl cyclase; (2) receptor coupling to K^+ channels; (3) receptor coupling to Ca^{2+} channels; (4) receptor coupling to protein phosphatases. Studies correlating SSTR inhibition of adenylyl cyclase with endogenous expression of G_i subunits in different strains of CHO cells, as well as the ability of antiserum to G_i or antisense G_i plasmids to block SSTR inhibition of adenylyl cyclase in AtT-20 and GH_4C_1 cells suggest the involvement of G_{i1}, and G_{i2} in mediating SSTR coupling to adenylyl cyclase (78-81). The additional involvement of G_{i3} in inhibiting forskolin stimulated adenylyl cyclase has also been demonstrated in GH_4C_1 cells (82). SSTRs are coupled to several subsets of K^+ channels (delayed rectifier, inward rectifier, ATP sensitive K^+ channels and large conductance Ca^{2+} activated BK channels) (83-86). Reconstitution experiments in GH_3 cells have indicated that G_{i3} couples SSTRs to K^+ channels (87). Receptor activation of K^+ channels causes reversible hyperpolarization of the membrane leading to the cessation of spontaneous action potential activity and secondary reduction in intracellular $Ca^{2+}{}_i$ due to inhibition of the normal depolarization induced Ca^{2+} influx *via* voltage sensitive Ca^{2+} channels (84, 88). In addition to this indirect effect on Ca^{2+} entry, SSTRs act directly on high voltage dependent Ca^{2+} channels *via* Goα2 protein to block Ca^{2+} currents (89, 90). Stimulation of both K^+ and Ca^{2+} channels may also occur through dephosphorylation of the channel proteins secondary to SSTR activation of a serine, threonine phosphatase (86). Furthermore, SSTRs may inhibit Ca^{2+} currents through induction of cGMP which activates cGMP protein kinase with further phosphorylation dependent inhibition of Ca^{2+} channels (91). SSTRs activate a number of phosphatases such as the serine threonine phosphatases, the Ca^{2+} dependent phosphatase calcineurin, and protein tyrosine phosphatases (PTP) (Fig. 3) (34, 35, 86, 92). This action is dependent on activation of pertussis toxin sensitive G proteins but the nature of the G proteins involved and whether they couple SSTRs directly or indirectly to phosphatases is unknown. Two other less well characterized signalling pathways for endogenous SSTRs have been described. The first is a Na^+/H^+ exchanger coupled to SSTRs *via* a pertussis toxin insensitive mechanism (93). The second is the phospholipase A2 (PLA-2) lipoxygenase pathway (94). Activation of PLC/IP3 is not thought to be a major signalling pathway for endogenous SSTRs (95).

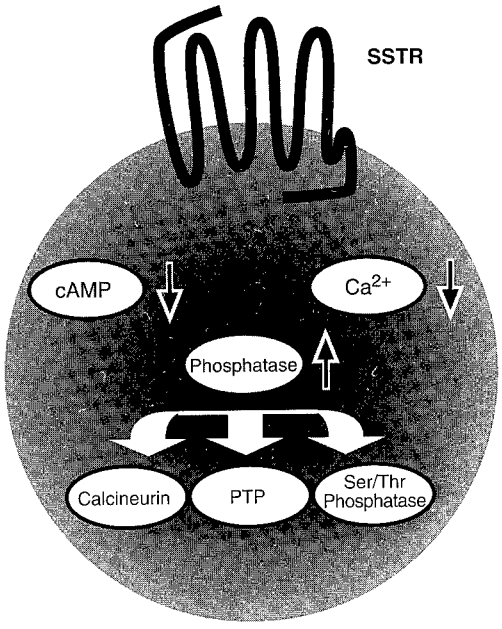

Fig. 3 - *Schematic depiction of the key second messenger systems modulated by SSTRs. Receptor activation leads to a fall in intracellular cAMP (due to inhibition of adenylyl cyclase), a fall in Ca^{2+} influx (due to activation of K^+ and Ca^{2+} ion channels) and stimulation of phosphatases such as calcineurin (which inhibits exocytosis), phosphotyrosine phosphatases (PTP-1C, which dephosphorylates growth factor receptor kinases to inhibit mitogenic signalling and cell proliferation) and serine threonine phosphatases (which dephosphorylate and activate Ca^{2+} and K^+ channel proteins).*

Coupling of Cloned SSTR Subtypes

Several laboratories have investigated the coupling of individually expressed hSSTR subtypes to G proteins and various effectors (Table 3). A putative consensus sequence for G protein coupling exists

Table 3 - *Membrane signalling pathways linked to cloned SSTRs.*

EFFECTOR	SSTR1	SSTR2A	SSTR2B	SSTR3	SSTR4	SSTR5	REF.
AC	↓	↓	↓	↓	↓	↓	14,19, 97
PTP	↑	↑		↑	↑		99-101
Ca^{2+} channels		↓					104
Na^+ /H^+ exchanger	↑					↓	103
PLC/IP_3		↑				↓↑	99,107
PLA_2					↑		106
MAP kinase					↑		

AC, adenylyl cyclase; PTP, phosphotyrosine phosphatase; PLC, phospholipase C; IP_3, inositol triphosphate; PLA_2, phospholipase A_2; MAP kinase, mitogen activated protein kinase.

in the third cytoplasmic loop of all SSTRs except SSTRI. Purified or expressed SSTR2 is capable of associating with the three G protein subunits, G_{i2}, G_{i3}, and G_{o2} (78, 79, 96). The nature of the G protein interactions for the other subtypes is unknown. Reports of the coupling of individual SSTR subtypes to adenylyl cyclase have been somewhat controversial. This is perhaps a reflection of the lack of appropriate G proteins in the host cells used for transfecting SSTRs or to the level of SSTR expression which may influence the fidelity of receptor G protein coupling. Overall, there is now a growing consensus that all five SSTR subtypes, and certainly the human isoforms are functionally coupled to inhibition of adenylyl cyclase *via* pertussis toxin sensitive G proteins (14, 19, 97). SSTR1, 2, 3, 4 have also been reported to stimulate PTP through pertussis toxin sensitive pathways (99-101). In the case of SSTR5, the rat receptor does not stimulate PTP in CHO-K1 cells (99), although there is preliminary evidence that the human isoform is capable of mediating PTP-dependent antiproliferative signalling in these cells (102). SSTR1 stimulates a Na^+/H^+ exchanger in transfected mouse fibroblast Ltk^- cells. This effect occurs *via* a pertussis toxin insensitive mechanism and does not require the third intracellular loop, the site of G protein coupling in many GPCRs. SSTR2 does not activate the Na^+/H^+ exchanger whereas coupling of subtypes 3, 4, and 5 to the exchanger is unknown (103). SSTR2 inhibits Ca^{2+} entry in Rin M5f cells by suppressing voltage-dependent Ca^{2+} channels (104). Likewise, SST inhibits voltage activated Ca^{2+} channels in cultured rat amygdaloid neurons (105). The modulation involves N- and P/Q-type Ca^{2+} channels and displays the pharmacological profile of the SSTR2 subtype (105). SSTR4 activates PLA2-dependent arachidonate production as well as MAP kinase cascade inCHO-KI cells, both *via* a pertussis toxin sensitive G protein (106). SSTR modulation of the PLC-IP_3 pathway remains controversial. In transfected CHO-KI cells SSTR5 inhibits IP_3 mediated Ca^{2+} mobilization whereas SSTR4 is without effect (99, 106). By contrast in COS-7 cells, SSTR5 and SSTR2A, both stimulate IP_3 production albeit at high agonist concentrations (107). These results mark only the beginning of the characterization of signal transduction pathways associated with SSTR5 and suggest that individual subtypes can activate multiple transmembrane effectors. Major voids still remain in our understanding of SSTR subtype selectivity for K^+ and Ca^{2+} channel coupling and of the molecular signals in the receptors responsible for activation of various phosphatases. Much of our current knowledge on subtype selectivity for signalling is based on transfected cells and should be interpreted with caution given the limitations of these systems. The emergence of selective agonists and antagonists should greatly facilitate the study of subtype selective effector coupling of endogenous SSTRs in normal cells.

Signal Transduction Pathways For SSTR Inhibition of Secretion and Cell Proliferation

Blockade of secretion by SSTR is in part mediated through inhibition of Ca^{2+} and to a lesser degree cAMP. Additionally, however, SST can inhibit hormone secretion stimulated by cAMP, Ca^{2+}, and other second messengers as well as in permeabilized cells (in which membrane ion channels are short circuited) through a distal effect which is believed to be mediated *via* a G protein dependent mechanism linking the receptor to exocytotic vesicles (92, 108, 109). Such direct inhibition of exocytosis is induced through SSTR dependent activation of the protein phosphatase calcineurin (92). The profound ability of SSTRs and other in-

hibitory receptors, e.g. α-adrenergic and galanin receptors to block secretion *via* this distal mechanism suggests that phosphorylation-dephosphorylation events rather than the Ca^{2+} signal play a key role in the distal steps of exocytosis (92). The specific SSTR subtypes involved in this process remain to be determined. The antiproliferative effects of SST are mediated both indirectly through inhibition of hormones and growth factors which promote cell growth, as well as directly *via* SSTRs present on target cells to inhibit mitogenic signalling of growth factor receptor kinases leading to growth arrest and induction of apoptosis. Several SSTR subtypes and signal transduction pathways have been implicated. Each of the three key second messenger systems modulated by SSTRs, cAMP, Ca^{2+} and PTP could be involved. Most interest is focused on a SST sensitive PTP shown to dephosphorylate and inactivate growth factor receptor kinase activity (34, 35, 110). A 66 kDa SH_2 domain containing PTP-1C has been shown to associate with membrane SSTRs and appears to translocate from the cytosol to the plasma membrane upon SSTR activation (35, 111). PTP-1C associates with both ligand occupied as well as unoccupied SSTRs (35). Activation of membrane associated PTP by SST, however, does, not appear to result from direct stimulation of the enzyme but rather from the recruitment of intracellular PTP to the membrane in a G protein dependent manner (111). In addition to a PTP mediated cytostatic action, SST affects cell cycle progression and apoptosis as first described by Pagliacci et al. (36). Subsequent studies by Srikant have shown that apoptosis occurs only when the cells are progressing through cell cycle division (112). Furthermore, apoptosis is signalled uniquely through the SSTR3 subtype and is associated with induction of P_{53} resulting from a dephosphorylation dependent conformational change (113). Overall, this means that in cells subjected to mitogenic stimulation, activation of appropriate SSTRs will lead to recruitment of PTP to the membrane resulting in dephosphorylation of receptor tyrosine kinases, attenuation of the mitogenic signal, and growth arrest. In cells that are committed to division and which express the SSTR3 subtype, SST will induce apoptotic cell death probably beyond the G_i checkpoint in S phase where apoptosis is initiated. The molecular signals in SSTR3 that confer subtype selectivity for apoptosis as well as the downstream pathways remain to be unravelled but could also involve PTP-mediated modulation of P_{53} and downstream genes implicated in cell cycle progression.

TISSUE EXPRESSION OF SSTR SUBTYPES

Expression of SSTRs in Normal Tissues

The pattern of expression of individual SSTRs has been studied at the mRNA level mainly in rodent tissues using various techniques (14, 15, 53, 114-117). These studies have revealed expression of multiple SSTR mRNAs in brain and peripheral organs with an overlapping but characteristic pattern that is subtype selective, tissue-specific, and species-specific (reviewed in ref. 14). All 5 SSTR mRNAs occur in cerebral cortex, striatum, hippocampus, amygdala, olfactory bulb, and preoptic area. Areas rich in expression of individual subtype genes are cerebral cortex for types 1 and 2, amygdala for types 1 and 3, and the hypothalamus and preoptic area for type 5. Overall, SSTR4 is relatively poorly expressed in all brain regions compared to the other 4 subtypes. Likewise, there is a differentially weaker expression of all five SSTR genes in the brain stem compared to the higher brain regions. Interestingly, the cerebellum which generally expresses few SSTR binding sites is rich in SSTR3 mRNA. In contrast to the rat, there is negligible expression of SSTR5 mRNA in the human brain suggesting a striking species-specific distribution (118). With the recent availability of rabbit polyclonal SSTR antipeptide antibodies, attempts have now begun to localize SSTR subtype proteins by immunocytochemistry (119-120). With this method, the regional and cellular distribution of SSTR2A has been mapped in detail in the rat brain and shows expression of receptors in neuronal perikarya, dendrites, and axon terminals especially in cerebral cortex and limbic structures suggesting that this subtype may be involved in mediating SST effects on cognition, learning, and memory (119). Adult rat pituitary features all five SSTR genes whereas the adult human pituitary expresses four of the subtypes 1, 2, 3, and 5 (114, 121-123). hSSTR4 is transiently expressed during development but is absent in the adult (123). Since the whole pituitary consists of many different cell types, the question arises whether different pituitary cell subsets express different SSTR subtypes or whether multiple SSTR subtypes are coexpressed in single cells (120). Two recent studies have addressed this question using either double-labelling *in situ* hybridization analysis or colocalization by combined *in situ* hybridization (for SSTR mRNAs) and immunocytochemistry (for pituitary hormones) (121, 122). Day et al. analysed SSTR 2 and 5 mRNA by dual label *in situ* hybridization and found SSTR5 mRNA in 70% of somatotrophes, 57% of thyrotrophes, 38% of corticotrophes, 33% of lactotrophes, and 21% of gonadotrophes (121). mRNA

for SSTR2 occurred in 40% of somatotrophes, 36% of thyrotrophes, 26% of lactotrophes, 3% of corticotrophes, and 8% of gonadotrophes (121). Not only were more cells positive for SSTR5 mRNA in this study, but the amount of mRNA per cell was also higher for SSTR5 than SSTR2. O'Carroll and Krempels examined the expression of mRNA for the five SSTRs in rat pituitary cells and found all five genes in each of the major pituitary cell subsets with high level of SSTR4 and 5 in somatotrophes and SSTR2 mRNA in thyrotrophes (122). These investigators found that the maximum number of cells in any pituitary cell subpopulation that was positive for a given SSTR mRNA was only ~ 20%, with no selectivity in the pattern of expression of mRNA for SSTR2 or SSTR5 (122). The colocalization strategy used consisted of immunohistochemistry for the pituitary hormones followed by *in situ* hybridization, and probably accounts for the poor expression of SSTR mRNA, since the immunostaining procedure before *in situ* hybridization leads to mRNA loss and underestimation of the number of SSTR mRNA containing cells (122). The results from both studies demonstrate the expression of SSTR genes in all of the major pituitary cell types, including corticotrophes and gonadotrophes previously thought to be SSTR negative. The finding by Day et al. that SSTR5 mRNA is more abundant than SSTR2 mRNA throughout the pituitary is consistent with the higher potency of SST-28 than SST-14 for inhibiting GH and TSH secretion and with the binding selectivity of pituitary membrane SSTRs for SST-28 compared to SST-14 (3, 25). SSTR5 also appears to be the principal subtype mediating GH and TSH suppression in the human pituitary based on pharmacological evidence correlating the preferential agonist binding properties of some SST analogs for hSSTR2 and hSSTR5 with their ability to regulate pituitary hormone secretion from human fetal pituitary cultures (64).

As in the brain, the five SSTR mRNAs are variably expressed in peripheral tissues (14, 15, 53, 114). Rat pancreas displays weak expression of only one SSTR subtype mRNA, that for SSTR2 (114). Preliminary evidence based on reverse transcriptase PCR has shown expression of mRNA for each of the five subtypes in whole rat islets with the following relative abundance: SSTR2 > SSTR1 = SSTR4 > SSTR3 = SSTR5 (124). Given the known selectivity of SST-14 for inhibiting glucagon and of SST-28 for suppressing insulin, together with earlier binding data showing preferential expression of SST-14 sites on α cells and of SST-28 sites on β cells, the low level expression of SSTR5 mRNA in whole islets comes as a surprise since being the only SST-28 selective subtype, one would predict this isoform to be the predominant β cell subtype (125). Future studies using *in situ* hybridization and/or receptor immunocytochemistry on single islet cells will hopefully provide the much needed information on the pattern of expression and function of the five SSTRs in different islet cell populations. In rat stomach SSTR1-4 mRNA has been identified by ribonuclease protection assay whereas RT-PCR yielded SSTR2-5 mRNA (14, 114, 126). Human stomach expresses all 5 subtypes (126). Rat small intestine displays moderate levels of SSTR1 and 5, low levels of SSTR3 and 4 but no expression of SSTR2 mRNA (14, 114). The adrenals, a known target of SST action, display a rich concentration of SSTR2 and modest levels of SSTR1 and 3 (14, 114). Some of the other peripheral organs exhibit a surprising level of expression of some SSTR subtypes, e.g. SSTR3 in the liver and spleen, and SSTR4 in the lung and heart (14, 114).

Expression of SSTRs in Tumors

The ability of SST to block hormone secretion led to the application of longacting SST analogs such as SMS in the early 80's for the treatment of hormone oversecretion from pancreatic intestinal and pituitary tumors (21, 127). A number of subsequent developments have led to mounting interest in the expression and function of SSTRs in many different forms of cancer. These include (i) the discovery that common solid tumors of breast, stomach, colorectal, pancreatic, lung, prostate, and cervical origin are rich in SSTRs; (ii) Shrinkage of pituitary and intestinal tumors with Octreotide treatment; (iii) the discovery of a direct tumoricidal effect of SST analogs on tumor cell lines; (iv) the ability to visualize and monitor SSTR positive tumors by *in vivo* receptor scintigraphy; and (v) the possibility of selective ablation of SSTR positive tumors and their metastases by targetted radiation using SST radioligands. Whilst the precise function of SSTRs in tumors is unclear, their presence can be exploited for diagnosis and therapy. To accomplish this, it is necessary to characterize the pattern of expression of tumor SSTRs in order to target the subtypes involved with appropriate agonists.

Expression of SSTR subtype mRNAs in pituitary tumors has been reported by three different groups whose collective results from 74 patients show that most pituitary tumors whether secretory or nonfunctional express at least one and typically multiple SSTR subtypes (Table 4) (123, 128-130). Since pituitary tumors are monoclonal in origin, this suggests the presence of more than one SSTR subtype in single tumor cells as also seems to be the

Table 4 - *Expression of mRNA for hSSTR1-5 in tumors.*

	EXPRESSION (%)					
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	REF
GH adenoma † (n=18-20)	61	95	44	5	86	123,128-130
Prolactinoma † (n=17-19)	84	63	35	6	71	123,128-130
Corticotroph adenoma † (n=7-9)	56	67	25	0	86	123,128-130
Nonfunctioning pituitary adenoma † (n=23-24)	38	75	43	13	48	123,128-130
Carcinoid (n=15)	87	93	53	40	NT	131
Insulinoma (n=4)	100	75	50	100	0	132
Glucagonoma (n=2)	100	100	100	100	0	132
Phaeochromocytoma (n=8)	100	100	100	100	100	133
Phaeochromocytoma (n=3)	100	100	0	0	0	132
Breast carcinoma (n=55)	73	98	72	35	NT	131
Breast carcinoma (n=50)	71	77	60	60	100	17
Renal carcinoma (n=13)	85	100	0	46	NT	131
Meningioma (n=14)	86	100	54	50	71	134
Glioma (n=7)	100	100	66	71	57	134

NT, not tested. † The no. of tumors analysed for the different subtypes differed slightly in the pooled data shown.

case for normal pituitary cells. SSTR2 and 5 which are both sensitive to octapeptide analogs are expressed the most frequently whereas SSTR3 and 4 are much less common (Table 4). There is no difference in the pattern of expression of the five subtypes between the different tumor types. Small numbers of peripheral endocrine tumors have been analysed for SSTR mRNA expression by RT-PCR (Table 4). Of 15 carcinoid tumors examined for SSTR1-4 mRNA expression, approximately 90% were positive for subtypes 1 and 2 and ~ 50% for SSTR3 and 4 (131). The small number of insulinomas and glucagonomas that have been studied have all been shown to be positive for SSTR1 and 4 mRNA, and 5/6 and 4/6 respectively also positive for SSTR2 and 3 mRNA, whereas SSTR5 was undetectable in this series (132). The majority of pheochromocytomas express all five SSTR mRNA species, SSTR2 and 4 mRNA being most abundant (133). Expression of SSTR 1-5 mRNA in breast cancer has been determined in over 100 samples by two groups (17, 131). All tumors analysed for SSTR5 mRNA were found to be positive for this subtype (17). The majority of tumors also expressed SSTR2 mRNA (17, 131). 60-70% expressed SSTR1 and 3 mRNA whereas SSTR4 mRNA was detectable in about ½ the tumors and was the least common subtype (17, 131). Of 13 renal cell carcinomas studied, the majority were positive for SSTR1 and 2 mRNA, 46% for SSTR4 mRNA, and 0% for SSTR3 mRNA. Meningiomas and gliomas are rich in SSTR2 mRNA expression (100%), SSTR1 is also an abundant subtype occurring in 90% of tumors followed by SSTR5 (67%), and SSTR3 and 4 (58%) (134). The general pattern that emerges from these studies suggests a very high frequency of SSTR2 mRNA expression in all tumors. SSTR1 mRNA is the next most common subtype followed by SSTR3 and 4. SSTR5 expression appears to be tumor specific being 100% positive in some tumors, e.g. breast, but absent in others, e.g. islet cell tumors. It should be noted that all of these studies are based on RT-PCR analysis of RNA extracts from tumor samples and cannot distinguish between the occurrence of receptors in tumor cells and those in peritumoral structures such as blood vessels and immune cells. Furthermore, it will be necessary to correlate mRNA expression with receptor protein expression by immunocytochemistry or binding analysis with subtype selective analogs since mRNA in tumors may not necessarily reflect receptor protein expression.

REGULATION OF SSTR SUBTYPES

Agonist-Dependent SSTR Regulation and Desensitization Response

Although the acute administration of SST-14 or SMS produces a diverse range of biological effects, the initial response diminishes with continued exposure

to the peptides due to the development of tolerance (21). Patients with SST producing tumors display sustained hypersomatostatinemia which, however, causes minimal symptomatology notably mild steatorrhea, diabetes mellitus, and cholelithiasis secondary to inhibition of pancreatic exocrine secretion, insulin release, and gallbladder contraction (135). Long-term therapy with SMS is also accompanied by signs and symptoms related to these features (21). Most patients develop mild steatorrhea to which they become tolerant after 10-14 days. Likewise, they adapt rapidly to some of the other effects of SMS, e.g. inhibition of insulin and TSH secretion, and thereby develop minimal signs of carbohydrate intolerance or hypothyroidism. Some effects, however, do persist, e.g. inhibition of gallbladder emptying which gives rise to a significant increase in the incidence of cholesterol gallstones in 20-30% of patients after 1-2 years of treatment (21). What is most interesting is that hormone producing tumors such as GH adenomas, carcinoid and VIPomas continue to respond to SMS injections with persistent suppression of hormone secretion frequently for several years. This suggests a differential regulation of SSTRs in normal tissues and in tumors. Agonist-mediated receptor downregulation could explain the desensitization responses of insulin, TSH, and pancreatic exocrine secretion. The SSTRs involved in modulating biliary function are clearly different since they do not appear to downregulate with continued SST treatment. The ability of hormone secreting tumors to withstand desensitization may be due to several factors. Tumors express a high density of SSTRs compared to surrounding normal tissues as clearly observed by *in vivo* receptor imaging (21, 127). Conceivably, SSTRs in tumors behave differently due to a loss of normal receptor regulatory function or to an alteration in the pattern and composition of the various subtypes expressed or because of abnormal receptor signalling.

Agonist-specific desensitization is common to many GPCRs and is associated with receptor phosphorylation, uncoupling of the receptor from G proteins, receptor internalization and receptor degradation (136). The cytoplasmic C-tail sequence contains sites of phosphorylation on serine and threonine residues as well as tyrosine internalization signals and is critically important in these processes. Agonist-induced uncoupling of SSTRs from G proteins has been shown in AtT-20 cells (137). Agonist-dependent internalization of SSTRs has been demonstrated in rat anterior pituitary cells, in rat islet cells, and AtT-20 cells (138-140). Prolonged agonist exposure for 24-48 h has been shown to upregulate SSTRs in GH_4C_1 and Rin M5f cells (33, 141). Since normal pituitary and islet cells or their tumor cell derivatives express multiple SSTR subtypes, the response of individual SSTR species cannot be determined using these approaches (120-123). To circumvent this problem, several recent studies have characterized agonist regulation of SSTRs in stably transfected cells expressing individual SSTR subtypes. hSSTR2, 3, 4, and 5 undergo rapid internalization in a time and temperature-dependent manner over 60 min in CHO-K1 cells upon agonist activation (142). Maximum internalization occurs with hSSTR3 (78%) followed by hSSTR5 (66%), hSSTR4 (29%), and hSSTR2 (20%). In contrast, hSSTR1 failed to be internalized (142). Identical results have been reported for hSSTR1 and the mouse homolog of SSTR2 in transfected COS-7 cells (143). A different pattern of internalization has been described for the five rat SSTR subtypes in human embryonic kidney (HEK) cells in which rSSTR1 along with rSSTR2 and 3 undergo SST-14 or SST-28-dependent internalization; internalization of rSSTR5 is triggered only by SST-28 whereas rSSTR4 fails to be internalized in response to activation by either SST-14 or SST-28 ligands (144). The reason for this discrepancy is unclear but may be due in part to structural differences between the rodent and human SST isoforms as well as the cell lines used for transfecting the SSTRs. The effect of chronic agonist treatment for 22 h on membrane SSTR levels has been studied in the case of the five hSSTRs in CHO-K1 cells. Under these circumstances, hSSTR1 is upregulated by 110%, hSSTR2 and hSSTR4 by 26% and 22% respectively, whereas hSSTR3 and 5 show no change (142). Since the five SSTRs feature the sequence NPXXY at the junction of the VIIth TM and cytoplasmic tail similar to the NPXY internalization motif that has been implicated in mediating the internalization of a number of GPCRs through clathrin-coated pits, as well as several Ser and Thr phosphorylation sites in the C-tail and cytoplasmic loops, there are no obvious structural differences that can explain the differential ability or resistance of the five subtypes to undergo internalization (Fig. 1) (15, 17). Detailed mutagenesis studies will be required to identify the underlying molecular signals. Whilst there may be species differences in the rates of internalization of the various SSTR isoforms, certainly the five human SSTR subtypes appear to be dynamically regulated at the membrane by SST. The ability of SST to regulate hSSTRs may provide a mechanism for targetting selective subtypes for diagnosis and therapy. For instance, upregulation of hSSTR1 and 2 by appropriate agonist treatment could be used for en-

hancing SSTR expression for receptor scans. Subtypes such as hSSTR3 and 5 which are extensively internalized could be targetted with selective α or β emitting SSTR radioligands for radiotherapy of certain SSTR positive human cancers.

Regulation of SSTR Gene Expression

Expression of SSTR subtypes is tightly regulated during development and influenced by a variety of hormones as well as disease states. Steady state SSTR mRNA levels are augmented in response to treatment with cAMP, gastrin or EGF, and SST itself (53, 145, 146). Glucocorticoids regulate SSTR gene transcription in a time-dependent manner in GH4CI cells (147). Short-term exposure to glucocorticoids induces SSTRI and SSTR2 mRNA whereas more prolonged treatment inhibits transcription of both genes (147). Estrogen induces SST binding sites in cultured rat pituitary cells and upregulates mRNA expression of SSTR2 and SSTR3 in rat prolactinoma cells and of SSTR2 mRNA in breast cancer cells (148-150). Thyroid hormone induces mRNA for SSTRI and SSTR5 in mouse thyrotroph tumor cells (151). Starvation or insulinopenic diabetes is associated with decreased mRNA levels for SSTRI-3 in the pituitary, and of SSTR5 in the hypothalamus (152). The underlying mechanisms remain to be worked out. To gain a better understanding of extracellular and tissue-specific factors regulating SSTR genes, a number of laboratories have begun to analyse promoter sequences. rSSTRI, hSSTR2, rSSTR4, and hSSTR5 feature TATA-less, G+C-rich promoters typical of tissue-specific housekeeping promoters (153-157). In the case of hSSTR2, a novel initiator element SSTR2inr located close to the mRNA initiation site confers gene expression in the absence of the TATA box by binding a helix-loop-helix transcription factor SEF-2 which interacts with the basal transcription machinery (155). The four SSTR promoters that have been characterized contain consensus sequences for a number of common transcription factors. rSSTRI gene shows AP2 and Pit1 binding sites as well as a consensus TRE between -97 and -81 bp downstream from Pit1 (153). hSSTR2 and rSSTR4 display multiple API and AP2 sites which may confer cAMP responsiveness (154, 156). The hSSTR5 promoter is also cAMP-inducible and additionally contains an Alu repeat sequence 1.2 kb upstream ofthe ATG (157). Alu sequences are primate-specific and when present in the promoter region may disrupt gene regulation. Thus, the occurrence of the Alu-like sequence in the hSSTR5 gene may be one explanation for the limited tissue expression of this receptor in humans compared to rodents. In addition, DNA polymorphism involving variations in the length of the Alu-like sequences may be associated with disease. In this context, it is of great interest that hSSTR5 is located in close proximity to the polycystic kidney and tuberous sclerosis genes on chromosome 16p 13.3 where it forms part of a conserved syntenic region.

CONCLUDING REMARKS

In the five years since the first SSTR was cloned, great progress has been made towards characterizing the structure and molecular pharmacology of this receptor family with now five members. The rich pattern of expression of SSTRs throughout the brain and in peripheral tissues coupled with the potent biological effects which they elicit clearly suggest that SSTRs represent a major class of inhibitory receptors which play an important role in modulating higher brain function, the secretory process, cell proliferation, and apoptosis. Synthetic agonists of SST have been in clinical use for over 10 years and now occupy an important therapeutic niche both in the diagnosis and treatment of tumors. These are, however, first generation compounds which interact with only three of the five SSTR subtypes. The recent development of agonists that are selective for the SSTR1, 4 subclass as well as the first of the peptide antagonists, should greatly expand the scope of SST pharmacotherapy. Future studies will need to define the function of individual subtypes, the role of multiple subtypes in the same cell, the downstream signalling pathways responsible for growth arrest and apoptosis, and the mechanisms underlying differential desensitization responses in tumor cells compared to normal cells. Finally, a great deal needs to be learned about the biology of SSTR dysfunction in neurological, gastroenterological, and immunological disorders.

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EXPRESSION OF THE FIVE SOMATOSTATIN RECEPTOR (SSTR1-5) SUBTYPES IN RAT PITUITARY SOMATOTROPHES: QUANTITATIVE ANALYSIS BY DOUBLE-LABEL IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

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Abstract: Using quantitative double-label fluorescence immunocytochemistry and confocal microscopy, we have analysed the pattern of expression of SSTR1-5 in normal rat pituitary somatotrophes. Antipeptide rabbit polyclonal antibodies were produced against the extracellular domains of SSTR1-5. SSTR antigens were colocalized in GH positive cells using rhodamine conjugated secondary antibody for SSTRs and FITC-conjugated secondary antibody for GH. SSTR5 was the predominant subtype which was expressed in 86 ± 9.7% of GH cells followed by SSTR2 in 42 ± 6.4% of GH positive cells. SSTR4 and SSTR3 were modestly expressed in 23 ± 4.7% and 18 ± 3.2% of somatotrophes respectively whereas SSTR1 was the least expressed subtype occurring in only 5 ± 1.2% of somatotrophes. These results demonstrate variable expression of the 5 SSTRs in somatotrophes. The preponderance of the SST-28 preferring SSTR5 subtype correlates with the reported higher potency of SST-28 than SST-14 for inhibiting GH secretion.

Somatostatin (SST) was originally isolated from the hypothalamus as the tetradecapeptide SST-14 based on its ability to inhibit GH secretion from the pituitary (1). It has since emerged as a multi-functional peptide system with two principal bioactive products, SST-14 and SST-28, which act on a diverse array of endocrine, exocrine, neuronal, and immune cell targets to inhibit secretion, modulate neurotransmission, and regulate cell division (2,3). Both SST peptides are released into the hypothalamo-hypophysial portal blood and regulate GH secretion physiologically (4). SST-28 is 3-8 fold more potent than SST-14 for GH inhibition *in vitro* and for binding to pituitary membrane SSTRs, and overall is as effective as SST-14 as a physiological regulator of *in vivo* GH secretion (5-7). The actions of SST are mediated by a family of G protein coupled receptors with five known subtypes termed SSTR1-5 (3,8). SSTR1-4 bind SST-14 ≥ SST-28 whereas SSTR5 binds SST-28 > SST-14 (3). All five SSTR mRNAs are present in the whole rat pituitary (9). The cell-specific expression of individual SSTR subtypes has been characterized only at the mRNA level with results that are contradictory (10,11). Using double-labeling *in situ* hybridization analysis of mRNA for SSTR2 and 5 in individual pituitary cell subpopulations, Day et al found that mRNA for SSTR5 was present in 60-70% of GH and TSH producing cells and was overall more abundant than SSTR2 mRNA throughout the pituitary (10). O'Carroll and Krempels colocalized SSTRs in rat pituitary by combined *in situ* hybridization (for SSTRs) and immunocytochemistry (for pituitary hormones) (11). They found that the maximum number of cells in any pituitary cell population that was SSTR positive was only ~ 20% with no selectivity in the expression pattern of any SSTR mRNA with a particular cell group (11). Furthermore, the presence of SSTR mRNA in either study may not necessarily correlate with SSTR subtype protein expression. Accordingly, we have developed polyclonal antibodies against the extracellular domains of SSTR1-5 and have begun to characterize the pattern of expression of each of the five SSTR proteins in pituitary cell subsets by quantitative double-label fluoresence immunocytochemistry. We report our analysis of SSTR expression in rat somatotrophes and show that the SST-28 preferring SSTR5 subtype is the predominant SSTR isoform in this cell.

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MATERIALS AND METHODS

Cell Lines Expressing SSTRs: Stable CHO-K1 transfectants individually expressing hSSTR1-5 were prepared as described and cultured in Ham's F-12 medium containing 10% fetal calf serum and 400 μg/ml G418 (12).

Antibodies to SSTR1-5: We have previously reported the production and character-ization of antipeptide rabbit polyclonal antibodies to human (h) and rat (r) SSTR1 and 2 (12,13) and used a similar strategy for generating antisera to SSTR3, 4, 5. The following oligo-peptides corresponding to deduced sequences in the amino terminal segment or extracellular loop 3 of hSSTR1-5 were synthesized by solid phase: hSSTR1 - ^{49}GTLSEGQGS^{57}; SSTR2 - ^{35} EPYYDLTS ^{42} ; SSTR3 - ^{35}PSPAGLAVS ^{43}; SSTR4 - ^{285}VTSLDAT ^{291}; SSTR5 - ^{4}LFP/SA/LSTPS ^{11}. The sequences selected were identical or nearly identical between the human and rat SSTR isoforms (8). Oligopeptides were synthesized with a lysine addition at the NH_2 - terminus to allow covalent conjugation to keyhole limpet hemocyanin with glutaraldehyde. The resulting immunogens were used for immunizing New Zealand white rabbits. Anti SSTR activity in rabbit sera was screened by the ability to inhibit [^{125}I-LTT] SST-28 binding to membrane SSTRs and by immunocytochemistry of stable CHO-K1 cells individually transfected with hSSTR1-5. Monkey anti-rat GH antibody was a gift from the NIADDK.

Immunocytochemistry and Confocal Microscopy: Adult male CD rats (150-200 g) were perfused with paraformaldehyde, the pituitary removed, post-fixed for 2 h in 4% paraformaldehyde and embedded in paraffin. 5-10 μm pituitary sections were treated with 10% normal goat serum followed by incubation with antibodies to SSTRs (1:200) or GH (1:300) overnight at 4° C. After washing, sections were incubated for 1 h at 20° C with

rhodamine conjugated goat antirabbit IgG for SSTR localization or FITC conjugated goat antimonkey secondary antibody for GH. Sections were then washed, air dried, and mounted in Immunofluor. All fluorescent images were visualized on a Zeiss LSM 410 inverted confocal microscope equipped with an argon/krypton laser. Rhodamine signals were imaged by exciting samples with helium/neon (543 nm) laser on a photomultiplier after passage through FT510, FT560, and LP590 filter sets. Fluorescein signals were imaged by exciting samples with a 488 nm line from an argon or an argon/krypton laser and the resulting fluorescence was collected on a second photo multiplier after passage through FT510, FT560, and BP515-540 filter sets. The images were overlapped for colocalization of SSTRs in GH immunoreactive cells. Images were obtained as single optical sections taken from the tissue and averaged over 32 scans/frame. All images were archived on a Bernoulli multi-disc and printed on Kodak XL58300 high resolution color printer. To validate the specificity of SSTR immunoreactivity, the following controls were included: (i) preimmune serum in place of primary antibody; (ii) primary antibody absorbed with excess antigen; (iii) nontransfected CHO-K1 cells.

Quantitative Analysis: 250-300 GH positive cells were analysed for colocalization of each of the five SSTRs. The mean percent of somatotrophes positive for a given SSTR subtype was determined in 6 fields and the results presented as mean ± SE.

RESULTS

Figure 1 depicts confocal images of SSTR immunoreactivity in CHO-K1 cells stably transfected with hSSTR1-5. Cells expressing any one of the five SSTRs displayed positive fluorescence only when reacted with the corresponding SSTR primary antibody (panels A-E). There was no crossreactivity of any of the five SSTR antisera with another (nonhomologous) subtype. SSTR immunoreactivity in each instance was localized predominantly on the cell membrane and to a small extent intracellularly. The fluorescence was specific since it was not observed in nontransfected CHO-K1 cells (panels F-J), or when preimmune serum was substituted for SSTR primary antibody or when the primary antibody was absorbed with excess antigen (not shown).

Confocal images showing rhodamine immunofluorescent localization (in red) of SSTR1-5 in rat pituitary are presented in Fig. 2 (panels A-E). All five SSTRs are localized in the majority of pituitary cells where they appear as surface membrane and cytosolic protein. No immunoreactivity was observed in control sections reacted with preimmune serum (Fig. 1, K,L) or antigen absorbed antibody (data not shown). GH positive cells were identified in the same sections by fluorescein immunofluorescence (in green) and accounted for approximately 50% of the pituitary endocrine cells (Fig. 2, F-J). Overlapping the SSTR and GH immunofluorescent images revealed colocalization of SSTR in a proportion of somatotrophes (yellow orange color) (Fig. 2, K-O) with a differential subtype-selective pattern of expression for the five SSTR isoforms. SSTR5 was the predominant subtype and was

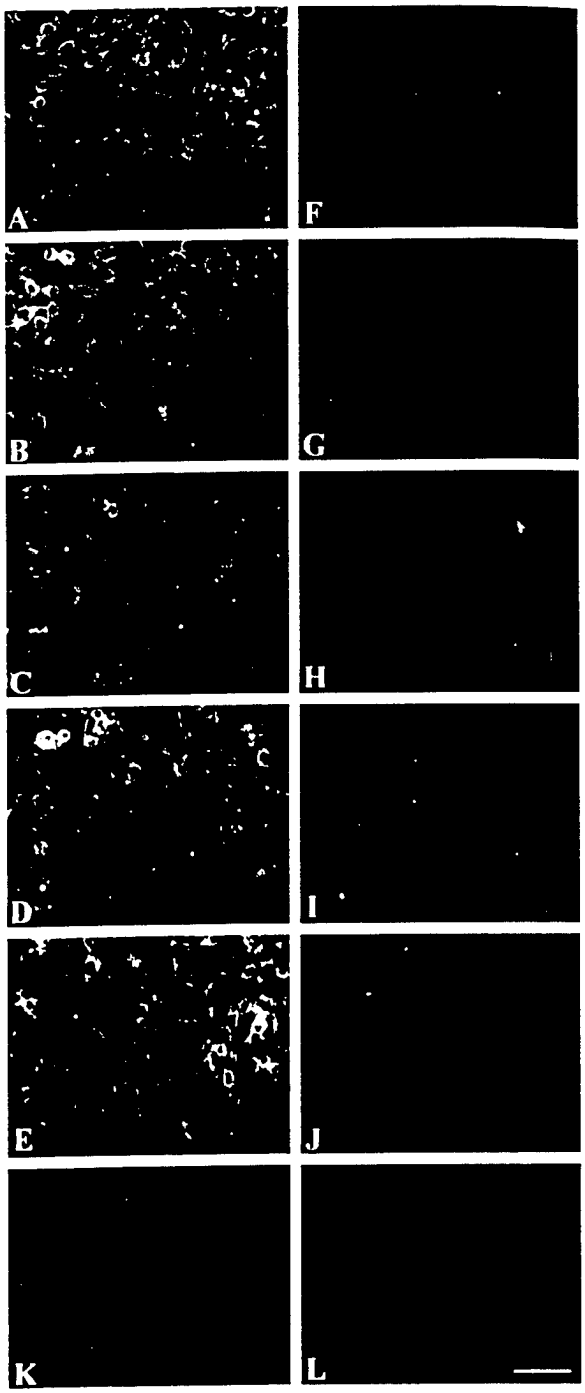

Fig. 1. Confocal images depicting SSTR immunofluorescence in CHO-K1 cells stably expressing hSSTR1-5. A-SSTR1, B-SSTR2, C-SSTR3, D-SSTR4, E-SSTR5. The corresponding control nontransfected CHO-K1 cells (F-J) are devoid of immunoreactivity. See text for description of panels K, L. Scale bar = 50 μM.

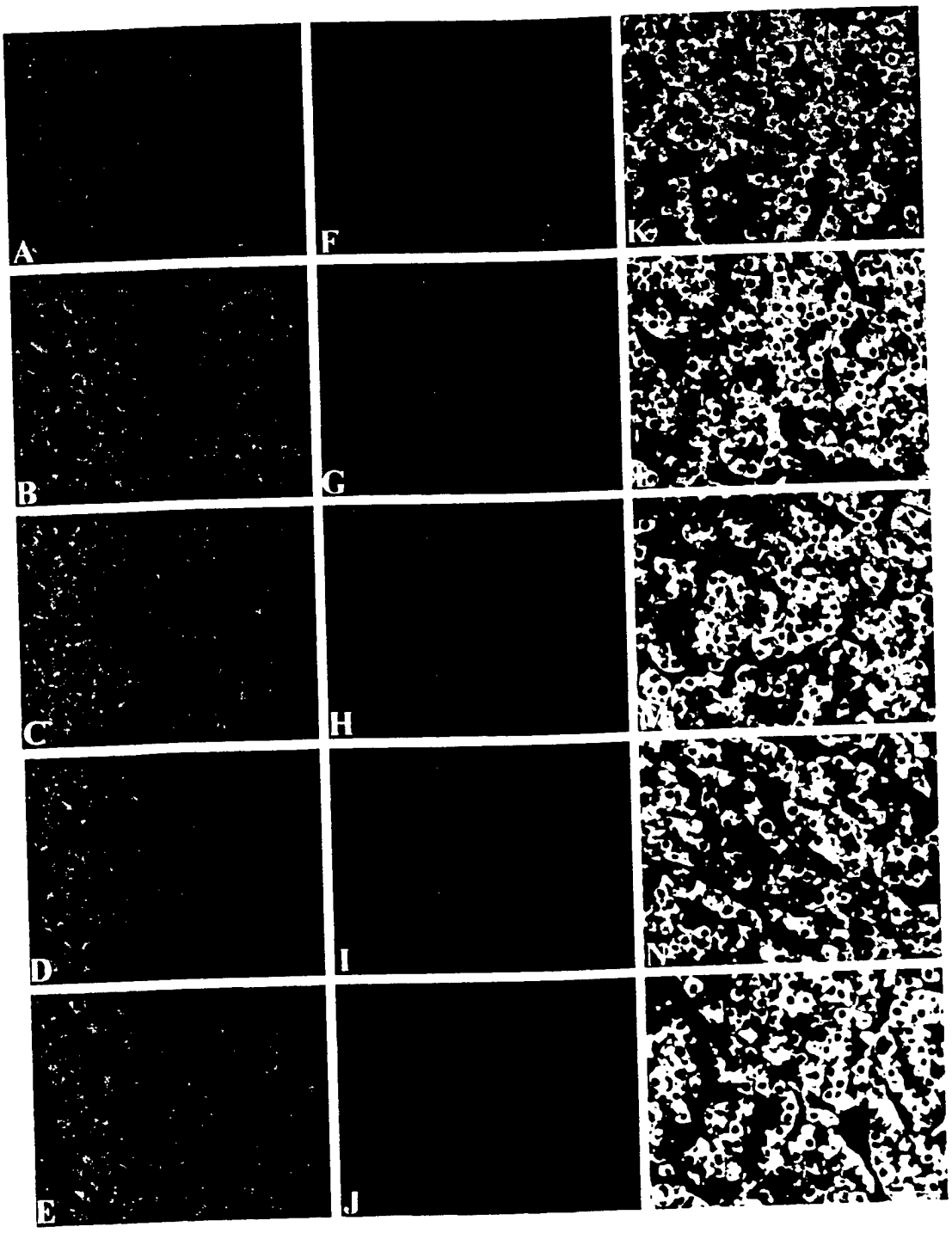

Fig. 2. Confocal images of pituitary cells double-labeled for immunofluorescence for SSTR1-5 and GH. SSTR1-5 immunoreactivity in separate pituitary sections is localized by rhodamine (red) fluorescence in panels A-E. GH positive cells in the same sections are identified by green immunofluorescence in panels F-J. Colocalization of SSTR1-5 (in red) in GH producing cells (in green) is indicated by a yellow-orange color in the overlapped images in panels K-O. Scale bar = 25 μM.

expressed in 86 ± 9.7% of GH cells followed by SSTR2 in 42 ± 6.4% of GH positive cells. SSTR4 and SSTR3 were modestly expressed in 23 ± 4.7% and 18 ± 3.2% of somatotrophes respectively whereas SSTR1 was the least expressed subtype occurring in only 5 ± 1.2% of somatotrophes.

DISCUSSION

In the present study we have produced and validated a panel of polyclonal antibodies against the five SSTRs. Using these antisera, we report the first immunohistochemical localization of all five SSTR protein isoforms in the normal rat pituitary. By fluorescence colocalization, we demonstrate variable expression of the five SSTRs in somatotrophes. SSTR5 is expressed in virtually all (~ 90%) GH producing cells and is the predominant subtype. SSTR2 is expressed in approximately half the somatotrophes whereas SSTR3 and 4 occur in ~ 20% of GH cells. SSTR1 is the least expressed subtype detectable in only a few of the GH producing cells. In the case of SSTR5 and 2, our immunohistochemical data are in close agreement with our earlier analysis of the distribution of mRNA for these two subtypes in 70% and 40% of somatotrophes respectively (10). Not only were more somatotrophes positive for SSTR5 mRNA in this study, but there was also a high abundance of SSTR5 mRNA/cell (10). The preponderance of SSTR5 (the only SST-28 selective SSTR) in somatotrophes correlates with the higher potency of SST-28 than SST-14 for inhibiting GH secretion and the binding selectivity of pituitary membrane SSTRs for SST-28 compared to SST-14 (5,6). Our results differ markedly from those reported by O'Carroll and Krempels who found a much lower incidence of mRNA for SSTR2 and 5 of 5% and 19% respectively in rat somatotrophes (11). The use of immunohistochemistry for the pituitary hormones before *in situ* hybridization in this study probably accounts for the poor expression of SSTR mRNAs since the immunostaining procedure before *in situ* hybridization leads to mRNA loss and underestimation of the number of SSTR mRNA containing cells (11). Although there are no direct data on the pattern of expression of SSTRs in the human pituitary, SSTR5 and SSTR2 appear to be the principal subtypes mediating GH suppression in the human based on pharmacological evidence correlating the preferential agonist binding properties of some SST analogs for SSTR5 and SSTR2 with their ability to regulate GH secretion from human fetal pituitary cultures (14).

The finding of SSTR5 in virtually all somatotrophes and the presence of additional subtypes 2, 3, 4 in 20-40% of these cells clearly indicates that individual somatrophes feature more than one SSTR isoform. An important property of somatotrophes is their ability to undergo rapid desensitization in response to agonist exposure (15). In this respect, it is interesting that somatotrophes display preferential expression of only those subtypes (SSTR2-5) which have been shown to undergo agonist-dependent internalization whereas they generally lack expression of SSTR1 which is resistant to agonist-mediated internalization (12).

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#3

INTERNATIONAL SYMPOSIUM
ON SOMATOSTATIN ANALOGS: BASIC
UPDATE AND CLINICAL PERSPECTIVES
Venice, October 24-25, 1997

SOMATOSTATIN RECEPTORS: SUBTYPE SELECTIVITY FOR DESENSITIZATION AND ANTIPROLIFERATION

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The diverse actions of somatostatin (SST) are mediated by a family of heptahelical G protein coupled receptors with five current members termed SSTR1-5. The human SSTR genes map to separate chromosomes and with the exception of SSTR2 are intronless. hSSTR1-4 display weak selectivity for SST-14 binding whereas hSSTR5 is SST-28 selective. Based on structural similarity and reactivity for octapeptide and hexapeptide SST analogs, SSTR2, 3, and 5 belong to a similar SSTR subclass; SSTR1, 4 react poorly with these analogs and belong to a separate subclass for which a prototypic agonist is the recently reported Rivier analog Des-AA^{125} [D-Trp^{8} I-Amp^{9}] SST-14. The binding domain of SST ligands is made up of residues in transmembrane domains III-VII and in the second extracellular loop. All five hSSTRs are functionally coupled to inhibition of adenylyl cyclase. Likewise, the five subtypes induce phosphotyrosine phosphatase 1-C (PTP-1C), in each case via pertussis toxin sensitive GTP binding proteins. Some of the subtypes are also coupled to K^+ and Ca^{2+} channels, to a N^+/H^+ hydrogen exchanger. to phospholipase-C and MAP kinase. mRNA for SSTR1-5 is widely expressed in brain and peripheral organs with an overlapping but characteristic pattern that is subtype selective, tissue-specific and species specific. Individual organs express multiple SSTR genes and in several cases like the brain, pituitary, and islet, all five SSTR genes. An important property of SSTRs is their ability to undergo agonist-dependent receptor regulation and to induce antiproliferation. This presentation highlights recent work from the authors' laboratory in both these areas with particular emphasis on subtype selectivity of the desensitization and antiproliferative responses.

Agonist-Dependent Receptor Regulation and Desensitization

Although the acute administration of SST produces a diverse range of biological effects, the initial effects diminish with continued exposure to the peptide due to the development of tolerance. On the other hand, hormone-producing tumors continue to respond to SST with persistant suppression of hormone secretion frequently for several years suggesting a differential regulation of SSTRs in normal tissues and tumors. Agonist-specific desensitization of G protein coupled receptors involves a series of discrete cellular steps which include receptor phosphorylation, uncoupling of the receptor from G proteins, receptor internalization and receptor degradation. The cytoplasmic C-tail contains sites for phosphorylation as well as tyrosine internalization signals and is critically important in these processes. Increasing evidence points to receptor internalization and signals events as independent functions of the receptors both requiring a conformational change that activates specific structural motifs. Internalization serves as an important component of the desensitization response and for

reactivating the receptor by dephosphorylating it and recycling it to the plasma membrane. To understand these processes, we have embarked on studies of agonist-induced receptor desensitization, internalization, or upregulation of the five hSSTR subtypes individually expressed in stable CHO-K1 cells. Cells were pretreated for 1 h with 10^{-6} mSST-14 (for SSTR1-4) and SST-28 (for SSTR5). Cells were then washed and tested for their ability of subsequently added agonist to inhibit adenylyl cyclase. In control cells, SST induced dose-dependent inhibition of forskolin-stimulated cAMP confirming functional coupling of all five receptors to adenylyl cyclase. Agonist pretreatment attenuated the potency of subsequently applied peptide to inhibit cAMP formation by all five receptors indicating receptor uncoupling from adenylyl cyclase. These results clearly demonstrate the ability of all five hSSTRs to undergo agonist-induced desensitization responses via G protein uncoupling. Uncoupling was most pronounced in the case of SSTR2 and 3 followed by hSSTR5, 1, and 4. To determine the role of receptor internalization in the desensitization response, cells were equilibrated overnight at 4° C with radioligand with or without excess cold SST-14 or SST-28 and then warmed at 37° C for different times to allow receptor internalization. hSSTR2, 3, 4, and 5 displayed rapid agonist-dependent internalization of radioligand in a time- and temperature-dependent manner over 60 min. Maximum internalization (78%) occurred with hSSTR3 followed by hSSTR5 (66%), hSSTR4 (29%), and hSSTR2 (20%). In contrast, hSSTR1 displayed virtually no internalization. Prolonged (24 h) treatment with 10^{-7} m agonist led to differential upregulation of some of the SSTRs. hSSTR1 was upregulated at the membrane by 110%, hSSTR2 and 4 by 26% and 22% respectively, whereas hSSTR3 and 5 showed little change. A hSSTR1-hSSTR5 chimera in which the C-tail of hSST1 was swapped by the C-tail of hSSTR5 was able to undergo significant 20% internalization confirming the presence of strong internalization signals in the C-tail of hSSTR5. Failure of hSSTR1 to internalize appears to be due at least in part to an absence of positive internalization signals and/or the presence of negative internalization signals in the C-tail. We next analysed by mutagenesis the cytoplasmic C-tail of hSSTR5 for its role in internalization and signalling. A series of deletion mutants were created to produce truncated receptors with variable length C-tail. Truncation of the C-tail to different lengths did not affect high affinity agonist binding. Progressive truncation of the C-tail of hSSTR5 produced both increased as well as decreased receptor internalization suggesting the presence of both positive and negative molecular internalization signals. The nature of these signals remains to be determined but could be on the several putative serine and threonine phosphorylation sites in the C-tail. The C-tail of hSSTR5 was also critical for receptor coupling to adenylyl cyclase. Whilst receptor internalization was both increased or decreased with progressive truncation of the C-tail, the efficiency of coupling to adenylyl cyclase was progressively diminished by shortening the C-tail. This suggests that internalization and signalling properties are independent functions of the receptor, both requiring the C-tail. Whereas internalization is residue specific, coupling to adenylyl cyclase is dependent on the length of the C-tail.

SSTR Expression in Breast Cancer and Subtype Selectivity For Antiproliferative Signalling

The antiproliferative effects of SSTR are mediated both indirectly through inhibition of hormones and growth factors which promote cell growth, as well as directly via SSTRs present on target cells to inhibit mitogenic signalling of growth factor receptor kinases leading to growth arrest and induction of apoptosis. We have determined mRNA expression of SSTR1-5 by semiquantitative RT-PCR in 66 primary human ductal NOS breast tumors. 93% were positive for at least one SSTR subtype. 89% expressed multiple SSTR mRNA; 32% were positive for all five SSTR mRNAs and 42% were positive for 3 or 4 SSTR mRNAs. hSSTR3 was expressed in 85% of tumors, hSSTR5 in 83%, hSSTR2 in 67%, hSSTR4 in 67%, and hSSTR1 in 58%. The relative amount of SSTR mRNA was highest for SSTR5 (8.3 fold vs actin), followed by SSTR1 (7.4), SSTR2 (7.2), SSTR3 (6.3), and SSTR4 (5.2). No correlation was found between SSTR expression, tumor size, lymph node status, or ER/PR status. However, there was a trend towards lower SSTR expression in high grade tumors and higher expression in low grade tumors. We have developed antipeptide rabbit polyclonal antibodies against the five SSTRs and have begun to analyse a subgroup of tumor samples by immunocytochemistry. Initial results show abundant expression of SSTR proteins in tumor cells as well as in peritumoral structures especially the muscle layers of blood vessels and in immune cells. The presence of SSTR mRNA generally correlates well with receptor protein expression.

Several SSTR subtypes and signal transduction pathways have been implicated in the antiproliferative effects of SST. Most interest is focused on a SST-sensitive PTP-1C shown to dephosphorylate and inactivate growth factor receptor kinase activity. We have shown that activation of membrane associated PTP by SST does not appear to result from direct stimulation of the enzyme but rather from the recruitment of intracellular PTP to the membrane in a G protein dependent manner. All five hSSTRs are capable of inducing PTP-1C. SSTRs, however, exhibit subtype selectivity for cytotoxic (apoptosis) and cytostatic (growth arrest) actions. Apoptosis occurs only when the cells are progressing through cell cycle division. It is signalled uniquely via hSSTR3 and is associated with induction of wild type p53 (secondary to dephosphorylation-dependent conformational change) and of Bax and endonuclease 2. By contrast, the other four SSTR subtypes signal cell cycle arrest with the following rank order: hSSTR5 > hSSTR2 > hSSTR4 > hSSTR1. These changes are associated with induction of the retinoblastoma protein pRB and the cyclin dependent kinase inhibitor p21. We have examined the nature of antiproliferative signalling by SST analogs in ER^+ and ER^- human breast cancer cell lines and shown that apoptosis is dependent on ER expression.

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#4

SYMPOSIUM NOVARTIS

THE EVOLVING ROLE OF SOMATOSTATIN ANALOGUES IN THE TREATMENT OF ACROMEGALY... SN1

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The advent of readily available peptidometic therapy for acromegaly has resulted in a new era of management options for pituitary tumors. Based upon an informed decision embodying potential risks and benefits, somatostatin analogs may be offered as primary therapy for acromegaly. As the response rate of micro-adenomas to somatostatin analogs may exceed that of surgery, primary medical management of these tumors may be warranted. In those patients who refuse surgery, cannot tolerate anesthesia or have other medical risks factors, somatostatin analogs are the safe and most effective treatment choices. Long-term treatment does not result in desensitization, and patients demonstrate enhanced sensitivity to the analogs with time. Availability of long-acting injectable analogs will certainly enhance patient convenience and compliance. Side effects of somatostatin analogs have been remarkably benign. Asymptomatic gallbladder echogenic events usually are confined to the first two years of therapy, and their frequency differs geographically. Development of receptor-subtype specific somatostatin analogs will allow discreet and selective hormonal and/or cell growth control by these novel compounds. As SSTRT2 and SSTR5 receptor subtypes appear to selectively mediate the somato-mammotroph lineage, their activation by highly selective peptide analogs offers a new dimension of GH and PRL control. SSTR2 specificity also appears to be a hallmark of thy2otroph cells. Conclusions : Despite the relatively high cost and requirement for three daily injections, patient compliance has been uniformly excellent. Somatostatin analogs are effective and safe in managing GH hypersecretion and resultant systemic effects of acromegaly.

SOMATOSTATIN RECEPTOR SUBTYPES: BASIC RESEARCH AND CLINICAL IMPLICATIONS SN2

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Human somatostatin receptors (hSSTRs) comprise a family of heptahelical membrane proteins encoded by five nonallelic genes localized on separate chromosomes. hSSTR1-4 display weak selectivity for SST-14 binding whereas SSTR5 is SST-28 selective. Based on structural similarity and reactivity for octapeptide and hexapeptide SST analogs, SSTR2,3, and 5 belong to a similar SSTR subclass ; SSTR1,4 react poorly with these analogs and belong to a separate subclass for which a prototypic agonist is the recently reported Rivier analog Des-AA^{1,2,5} [D-Trp^{8} I-Amp^{9}] SST-14. The binding domain for SST ligands is made up of residues in transmembrane domains III-VII and in the second extracellular loop. All five hSSTRs are functionally coupled to inhibition of adenylyl cyclase. Likewise the five subtypes induce PTP1-C in each case via pertussis toxin sensitive GTP binding proteins. All five hSSTRs undergo agonist induced G protein uncoupling and desensitization of cAMP responses. hSSTR2,3,4, and 5 display variable (20-80 %) subtype selective internalization upon agonist activation. hSSTR1 is not internalized but rather upregulated 2-fold at the membrane following 24 h agonist exposure. By double-label immunofluorescence confocal microscopy with SSTR antibodies, all pituitary cell subsets express multiple SSTR subtypes. Overall SSTR5 is the predominant pituitary subtype followed by SSTR2,1,3, and 4. Most tumors, e.g. pituitary, islet, carcinoid, breast, prostate, brain express at least one and typically multiple SSTR subtypes. SSTR2 is the most frequent and abundant isoform followed by SSTR1,3, and 4. SSTR5 expression appears to be tumor type-specific. SSTRs exert significant antitumor activity both by cytostatic and cytotoxic actions. hSSTR1,2,4,5 induce growth arrest whereas hSSTR3 uniquely signals apoptosis through induction of p53 and Bax. Finally, a novel role for SST has emerged as a putative neuroprotective agent in combatting excitotoxic cell death ofneurons exposed to NMDA agonists. The nature of the subtypes involved in this action await elucidation and may open the door to the application of suitable SST compounds for treating human neurodegenerative disorders.

SANDOSTATIN®: RELEVANCE OF PHARMACOKINETIC PROFILE IN THE MEDICAL TREATMENT OF PATIENTS SN3

Peter Marabach

Novartis Pharma Inc. 4002 Basle, Switzerland.

Sandostatin® (octreotide acetate) is available as an immediate release formulation for multiple daily subcutaneous injections, and a long-acting release formulation (Sandostatin® LAR®) for intramuscular injection every 4 weeks. The pharmacokinetic (PK) profile after subcutaneous Sandostatin® is characterized by peak-to-trough variations of 200 %, and an elimination half-life of 90 minutes, normally necessitating three daily injections. Growth hormone (GH) profiles are strongly and inversely correlated to octreotide plasma concentration profiles, i.e. immediately after the injection of Sandostatin, GH is suppressed for several hours, returning towards basal levels at the end of an injection interval. Pharmacokinetic/pharmacodynamic (PK/PD) evaluations reveal, that octreotide serum concentrations of 250 pg/mL lead to approx. 50 % occupancy of the somatostatin receptor compartment, corresponding to the IC_{50} in receptor-binding studies, and the EC_{50} in *in vitro* GH-release experiments.

#5

Subtype-Selective Induction of Wild-Type p53 and Apoptosis, but not Cell Cycle Arrest, by Human Somatostatin Receptor 3

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Somatostatin (SST) exerts direct antiproliferative effects in tumor cells, triggering either growth arrest or apoptosis. The cellular actions of SST are transduced through a family of five distinct somatostatin receptor subtypes (SSTR1-5). Whereas growth inhibition has been reported to follow stimulation of protein tyrosine phosphatase via SSTR2 or inhibition of Ca^{2+} channels via SSTR5 in heterologous expression systems, the subtype selectivity for signaling apoptosis has not been investigated. The tumor suppressor protein p53 and the protooncogene product c-Myc regulate cell cycle progression (growth factors present) or apoptosis (growth factors absent). The p53-induced G_1 arrest requires induction of p21, an inhibitor of cyclin-dependent kinases, whereas apoptosis requires induction of Bax. c-Myc is capable of abrogating p53-induced G_1 arrest by interfering with the inhibitory action of p21 on cyclin-dependent kinases. We have, therefore, investigated the regulation of p53, p21, c-Myc, and Bax and cellular apoptosis in relation to cell cycle progression in CHO-K1 cells stably expressing individual human SSTR1-5. We demonstrate that apoptosis is signaled uniquely through human SSTR3 and is associated with dephosphorylation-dependent conformational change in wild-type (wt) p53 as well as induction of Bax. The induction of wt p53 occurs rapidly and precedes the onset of apoptosis. We show that the increase in wt p53 is not associated with the induction of p21 or c-Myc when octreotide-induced apoptosis becomes evident, suggesting that such apoptosis does not require G_1 arrest and is not c-Myc dependent. These findings provide the first evidence for hormonal induction of wt p53-associated apoptosis via G protein-coupled receptor in a subtype-selective manner. (Molecular Endocrinology 10: 1688–1696, 1996)

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Molecular Endocrinology

INTRODUCTION

Somatostatin (SST) regulates the function of central and peripheral nervous systems, endocrine and exocrine organs, as well as vascular and immune systems. These actions are mediated by specific membrane SST receptors coupled to multiple signal transduction pathways, which elicit antisecretory as well as antiproliferative responses (1, 2). The growth inhibitory action of SST can lead to cell cycle block due to G_1 arrest and/or apoptosis (3–6). Although the ability of SST to signal growth arrest has been attributed to activation of phosphotyrosine phosphatase (PTP), which dephosphorylates and inactivates growth factor receptor kinases (7), it is not known whether growth arrest is necessary for induction of apoptosis. Five distinct SSTRs (SSTR1-5) have been cloned and classified into distinct subfamilies, one comprising hSSTR2, -3, and -5, and the other made up of human (h) SSTR1 and -4, on the basis of their ability or inability to bind octapeptide SST analogs (1, 2). SSTRs regulate a number of G protein-linked signaling pathways, including adenylyl cyclase (SSTR1-5), Ca^{2+} channels (SSTR2), phospholipase A_2 (SSTR4), PTP (SSTR1, -2, and -3), and mitogen-activated protein kinase (SSTR4) (1, 2, 8–10). Although growth inhibition is reported to be signaled in a subtype-selective manner involving either stimulation of PTP (SSTR2) or inhibition of Ca^{2+} channels (SSTR5) (9), the subtype selectivity for signaling apoptosis and the underlying mechanisms have not been elucidated.

The tumor-suppressor protein p53 triggers G_1 arrest in the presence of growth factors, but induces apoptosis, which is associated with an increase in Bax in cells deprived of growth factor stimulation (11). The ability of p53 to induce G_1 arrest requires the induction of p21 (also called WAF-1/CIP1/SDI1), an inhibitor of cyclin-dependent kinases regulating the G_1 check point (12). The protooncogene product c-Myc promotes cell cycle progression or apoptosis in the presence or absence, respectively, of survival factors and, moreover, is capable of abrogating p53-induced G_1 arrest by inducing an inhibitor of p21 (13–16). Inhibition

of mitogenic signaling by SST should, therefore, be conducive to the induction of apoptosis in a manner requiring p53 and/or c-Myc. We have established stably transfected CHO-K1 cells expressing individual hSSTR1-5 and have previously described their pharmacological characterization and functional coupling to adenylyl cyclase (17, 18). To elicit the subtype selectivity and signaling mechanism underlying SSTR-mediated apoptosis, we investigated the regulation of p53, p21, c-Myc, and Bax in relation to cell cycle progression and induction of apoptosis in these cells.

RESULTS

Octreotide (OCT) Induction of Cell Cycle Progression Is Signaled via hSSTR3

We analyzed cellular DNA stained with the intercalating dye propidium iodide (PI) by flow cytometry in CHO-K1 cells stably expressing individual hSSTR1-5 after 24-h incubation in the absence or presence of 10 nM OCT (hSSTR2, -3, and -5) or (D-Trp^8)SST-14 (hSSTR1 and -4). A significant 35% decrease in G_0/G_1 and a 2-fold increase in S phase were seen in OCT-treated cells expressing hSSTR3. By contrast, the DNA profile was not altered in cells expressing hSSTR2 and -5 incubated with OCT or in cells expressing hSSTR1 and -4 incubated with (D-Trp^8)SST-14 (Fig. 1). The ability of (D-Trp^8)SST-14 to regulate cell cycle progression in cells expressing hSSTR3 confirmed the subtype selectivity determined using OCT (not shown).

Induction of Wild-Type (wt) p53 by OCT Is Signaled via hSSTR3

In hSSTR3-expressing cells, OCT induced a 3-fold increase in p53-positive cells immunodetected with the monoclonal anti-p53 antibody pAb 1801 (which reacts with both wt and mutant conformations of p53; Fig. 2A). As seen in this figure, there was an increase in the fluorescence intensity of pAb 1801-reactive p53 after OCT treatment. By contrast, no induction of p53 was detected in cells expressing hSSTR2 and -5 treated with OCT or in cells expressing hSSTR1 and -4 treated with (D-Trp^8)SST-14. Furthermore, the intensity of p53 immunofluorescence detected using a mutant-specific antibody (pAb 240) in cells expressing hSSTR3 or other subtypes was not altered by peptide treatment (Fig. 2B). This suggests that OCT-induced, hSSTR3-mediated increase in p53 is wt conformation specific. SSTR3 selectivity for p53 induction was also confirmed using (D-Trp^8)SST-14 (not shown). The changes in cell cycle parameters and p53 in hSSTR3-expressing cells induced by OCT was dose dependent over a concentration range of 0.1–100 nM (Fig. 3A). Although the increase in p53 occurred in all phases of the cell cycle, there was a preferential accumulation of cells with elevated p53 in S phase at higher concen-

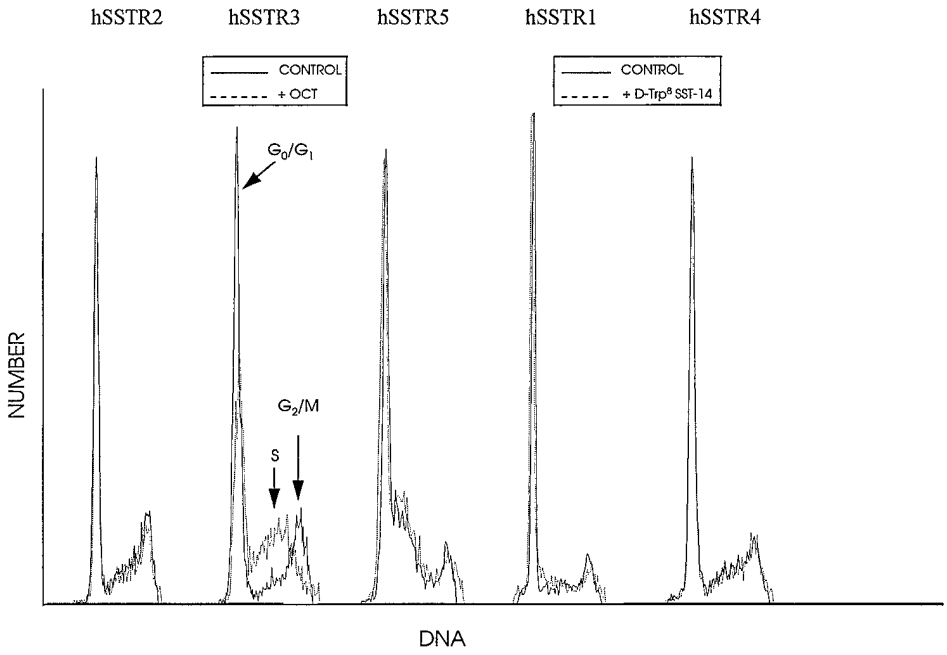

Fig. 1. Effect of OCT on Cell Cycle Parameters in CHO-K1 Cells Expressing hSSTR1-5

Representative plots depicting phase distribution of cells incubated for 24 h in the absence (*solid lines*) and presence of 10 nM OCT (hSSTR2, -3, and -5) or (D-Trp^8)SST-14 (hSSTR1 and 4; *dotted lines*). Cellular DNA was stained with PI and analyzed by flow cytometry. OCT induced a significant decrease in G_1 and an increase in S phase in cells expressing hSSTR3, but not in cells expressing other SSTR subtypes. The data shown are representative of four separate experiments.

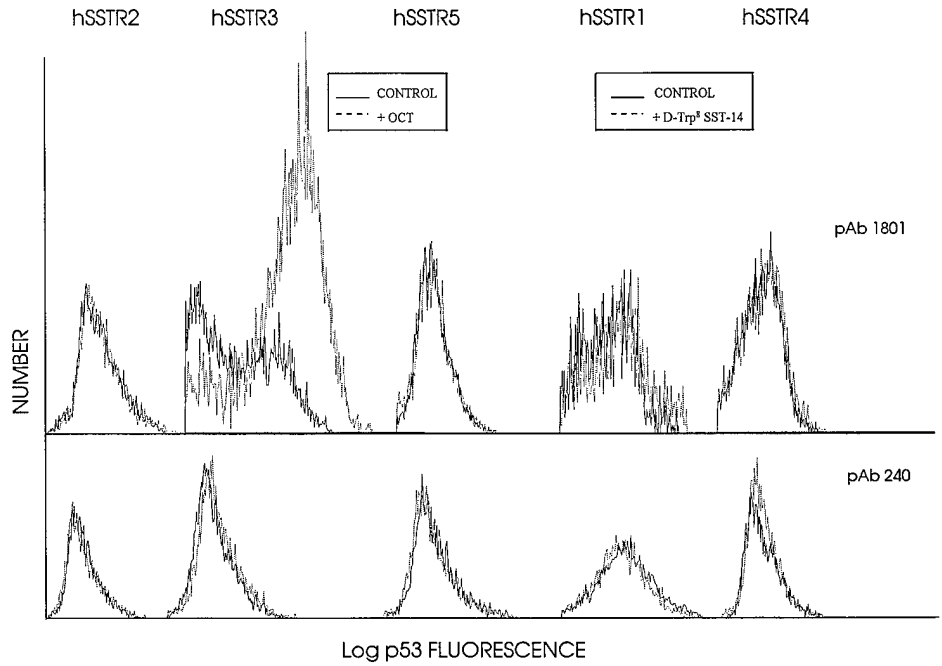

Fig. 2. A, Subtype-Selective Induction of wt p53 Is Signaled through hSSTR3

Representative plots depicting immunoreactive p53 detected using two epitope-specific anti-p53 antibodies in cells incubated for 24 h in the absence (*solid lines*) and presence of 10 nM OCT (hSSTR2, -3, and -5) or (D-Trp^8)SST-14 (hSSTR1 and -4; *dotted lines*). A 3-fold increase in immunoreactive p53 and a 100-fold increase in fluorescence intensity labeled with wt-specific pAb 1801 are seen in CHO-K1 cells expressing hSSTR3, but not in other hSSTR subtypes. B, p53 detected by the mutant-specific p53 antibody pAb 240 is not influenced by OCT in cells expressing all five hSSTRs. The data shown are representative of four separate experiments.

trations of OCT. To determine whether the OCT-induced increase in p53 is due to a change in the phosphorylation state of the molecule, we subjected the cell extracts to immunoblot analysis using the antibody pAb 1801. In control cells two distinct bands of p53 representing the phosphorylated form (top band) and the nonphosphorylated form (bottom band) were detected. In OCT-treated cells, there was a selective increase in the labeling of the band with greater mobility representing the dephosphorylated form of p53 (Fig. 3A, *inset*). Thus, the increase in p53 immunoreactivity detected by pAb 1801 was due to a dephosphorylation-dependent change resulting in wt conformation. Analysis of the cell cycle parameters revealed that OCT induced a dose-dependent decrease in G_0/G_1 and an increase in S phase (Fig. 3B). G_2 peak size increased 2-fold in cells incubated with 0.1 nM OCT, but decreased progressively with increasing peptide concentration. The decrease in the G_1/S ratio indicates that OCT does not invoke G_1 arrest via hSSTR3.

Induction of wt p53 Signaled through hSSTR3 Leads to Apoptosis

To determine whether the induction of p53 leads to apoptosis, cells expressing individual hSSTR subtypes were incubated in the absence or presence of 100 nM peptide for 24 h, and the DNA was extracted and subjected to pulse field gel electrophoresis. DNA degradation resulting in the formation of oligonucleosomal-sized fragments as a consequence of peptide treatment was seen in cells expressing hSSTR3, but not in cells expressing the other four subtypes (Fig. 4). In a separate experiment we compared the time course of induction of wt p53 and apoptosis in CHO-K1 cells expressing hSSTR3 during incubation with 100 nM OCT. Whereas the OCT-elicited increase in p53 was rapid, time dependent, and sustained, with a more than 2-fold increase within 1 h, DNA fragmentation was evident only after 24 h and became massive after 96 h (Fig. 5).

Induction of Apoptosis by OCT Does not Require Cell Cycle Arrest

Analysis of DNA staining by flow cytometry revealed that the decrease in G_1 peak in cells incubated with 100 nM OCT was accompanied by the formation of a distinct, hypodiploid, DNA peak. The number of cells in the hypodiploid region designated A_0 increased with time, and after 96 h, their greater hypodiploidicity due to profound DNA damage was reflected by the leftward shift of the peak (Fig. 6A). Induction of p21 by p53 results in G1 arrest, whereas concomitant activation of c-Myc can abro-

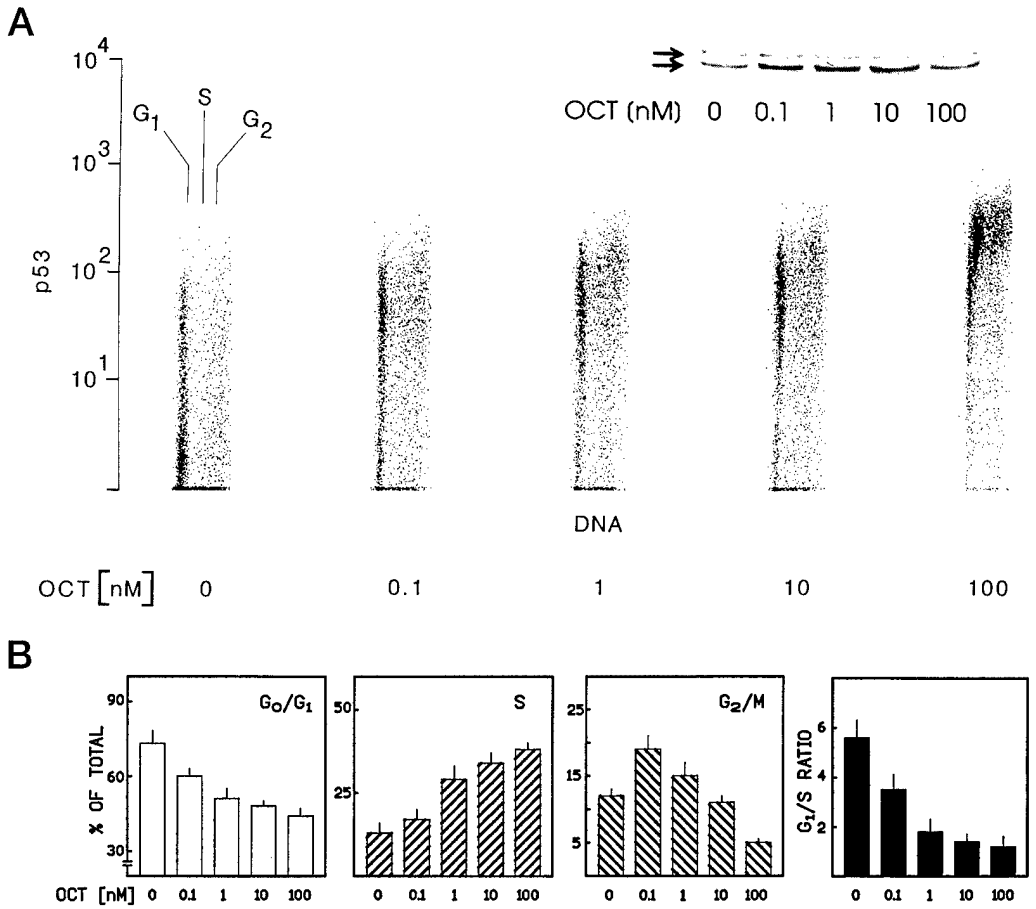

Fig. 3. Dose-Dependent Effect of OCT on p53 and Cell Cycle Parameters in hSSTR3-Expressing Cells

A, Bivariate analysis of dual labeled cells show that at low concentration (0.1 nM), an OCT-induced increase in p53 occurs in all phases of the cell cycle, whereas at higher concentrations, cells expressing high levels of p53 exhibit preferential accumulation in S phase. *Inset*, Western blot analysis of cell extracts, demonstrating an OCT-induced increase in the hypophosphorylated form of p53 in CHO-K1 cells. B, Effect of OCT on cell cycle distribution reveals a dose-dependent decrease in G_1 and an increase in S phase. The dose-dependent decrease in the G_1/S ratio shows the absence of G_1 arrest during OCT treatment. An increase in G_2 is seen only at lower doses of OCT. The number of cells in each phase is expressed as a percentage of the total number of cells analyzed in each sample (n = 4).

gate wt p53-mediated G_1 arrest and promote apoptosis (13, 16). To determine whether the failure of OCT-induced wt p53 to impose G_1 arrest is due to a lack of induction of p21 or to concomitant activation of p21 and c-Myc, we compared the levels of wt p53, p21, and c-Myc under these conditions. Induction of wt 53 by OCT was maximal by 24 h, as determined by the increase in wt p53 immunofluorescence detected with pAb 1801, in terms of both intensity and peak size (Fig. 6B). The p53 immunofluorescence remained high in cells treated with OCT for 96 h, although there was a decrease in peak size. By contrast, OCT failed to induce p21 at both time points (Fig. 6C). c-Myc levels remained unaltered in cells incubated with OCT for 24 h, but exhibited a delayed increase after 96 h of treatment (Fig. 6D). The changes in wt p53 levels and cell cycle distribution as well as the strong correlation between increased wt p53 content and apoptosis were evident upon bivariate analysis after simultaneous measurement of PI staining and pAb 1801 immunofluorescence in dual labeled cells (Fig. 6E). The population of cells in G_0/G_1 with low wt p53 content appeared to escape apoptosis initially during OCT treatment, but displayed significantly increased levels in subsequent cycle (compare 0 and 96 h, Fig. 6E). An increase in Bax was also seen in these cells during OCT treatment, and as in the case of wt p53, the increase was selective in cells with hypodiploid DNA content in the A_0 region (Fig. 6F). Immunocytochemical analysis of dual labeled cells revealed nuclear shrinkage and, furthermore, established that wt p53 is preferentially localized within the nucleus in OCT-treated cells in contrast to its distinct perinuclear localization in control cells (Fig. 7). As shown in Fig. 7, induction of wt p53 clearly preceded that of c-Myc, confirming the results obtained with flow cytometry.

Fig. 4. SSTR Subtype-Selective Induction of Apoptosis

CHO-K1 cells expressing individual hSSTRs were incubated for 24 h in the absence or presence of 100 nM OCT (hSSTR2, -3, and -5) or (D-Trp^8)SST-14 (hSSTR1 and -4). Field inversion gel electrophoresis of DNA extracts reveals that formation of oligonucleosomal fragments occurs only in hSSTR3-expressing cells after peptide treatment. The mobility of a 1-kilobase DNA ladder (Life Technologies) is shown in the first lane.

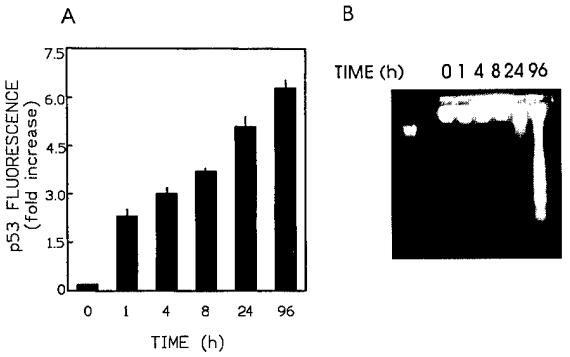

Fig. 5. Induction of p53 by OCT in hSSTR3-Expressing Cells Precedes the Onset of Apoptosis

A, Cells were incubated with 100 nM OCT for the indicated times, and pAb 1801-immunoreactive p53 was measured by flow cytometry. Results are expressed as the fold increase in fluorescence intensity compared with the control value (n = 4). The OCT-induced increase in p53 is rapid, time dependent, and sustained. B, Representative DNA gel electrophoresis, demonstrating that DNA damage resulting in oligonucleosome-sized DNA fragments occurs with a slower time course than that of the rapid induction of wt p53.

Induction of wt p53 and Apoptosis Signaled via hSSTR3 Is G Protein Dependent

To determine whether signaling of apoptosis via hSSTR3 is G protein dependent, we pretreated cells with pertussis toxin, which ADP ribosylates and inactivates G proteins known to mediate SSTR signaling. Pertussis toxin treatment reversed the OCT-elicited increase in wt p53 as well as the changes in cell cycle parameters and induction of apoptosis, as demonstrated by both flow cytometry and DNA fragmentation analysis (Fig. 8).

DISCUSSION

This study establishes that induction of apoptosis by SST analogs is signaled in a subtype-selective manner uniquely via hSSTR3. This requires the presence of wt p53 and its dephosphorylation-dependent conformational change. The induction of wt p53 by OCT occurs rapidly and precedes the onset of apoptosis. The hSSTR3-mediated induction of wt p53 and apoptosis occur in a pertussis toxin-sensitive, G protein-dependent manner. Additionally, our finding that neither p21 nor c-Myc increases initially coupled with the decrease in the G_1/S ratio demonstrate that OCT induction of wt p53 and apoptosis does not require cell cycle arrest in G_1 and is c-Myc independent. However, the delayed increase in c-Myc seen during prolonged OCT treatment raises the possibility that it may potentiate apoptosis at later stages. The present finding that wt p53-associated cell death elicited by OCT is signaled through hSSTR3 constitutes the first instance of induction of apoptosis by a G protein-coupled receptor in a subtype-selective manner.

Using epitope-specific anti-p53 antibodies, we have shown that the OCT-induced increase in immunoreactive p53 is wt conformation specific. The antibody pAb 1801 recognizes an antigenic site situated in the N-terminal region (located between amino acid residues 32–79) of wt p53 in its active as well as inactive conformations. Phosphorylation of Ser residues within this region (which occurs in growth-arrested cells) inhibits the binding of p53 to pAb 1801 (19), whereas the wt-specific conformational change induced by dephosphorylation of phosphoserine residues, increases its binding to this antibody (20). The increase in pAb 1801-immunoreactive p53 is due to OCT-induced dephosphorylation of Ser residues in wt p53, as evidenced by immunoblot analysis. Our findings also suggest that although cells containing the phosphorylated form of p53 are able to traverse initially through the cell cycle, they display an increase in the dephosphorylated form and almost complete loss of the phosphorylated form of p53 in subsequent cycles, as seen at 0 and 96 h, respectively (Fig. 6E). Its preferential intranuclear accumulation during OCT treatment combined with the present finding that its induction precedes the onset of apoptosis support a primary role for wt p53 in signaling OCT-induced apoptosis. The absence of G_1 arrest in these cells is similar to the finding by Adachi *et al.* in N417 small cell lung carcinoma cells undergoing wt p53-induced apoptosis (21).

We found that Bax, which promotes apoptosis, is induced during wt p53-associated apoptosis signaled via hSSTR3. Additionally, bivariate analysis of dual

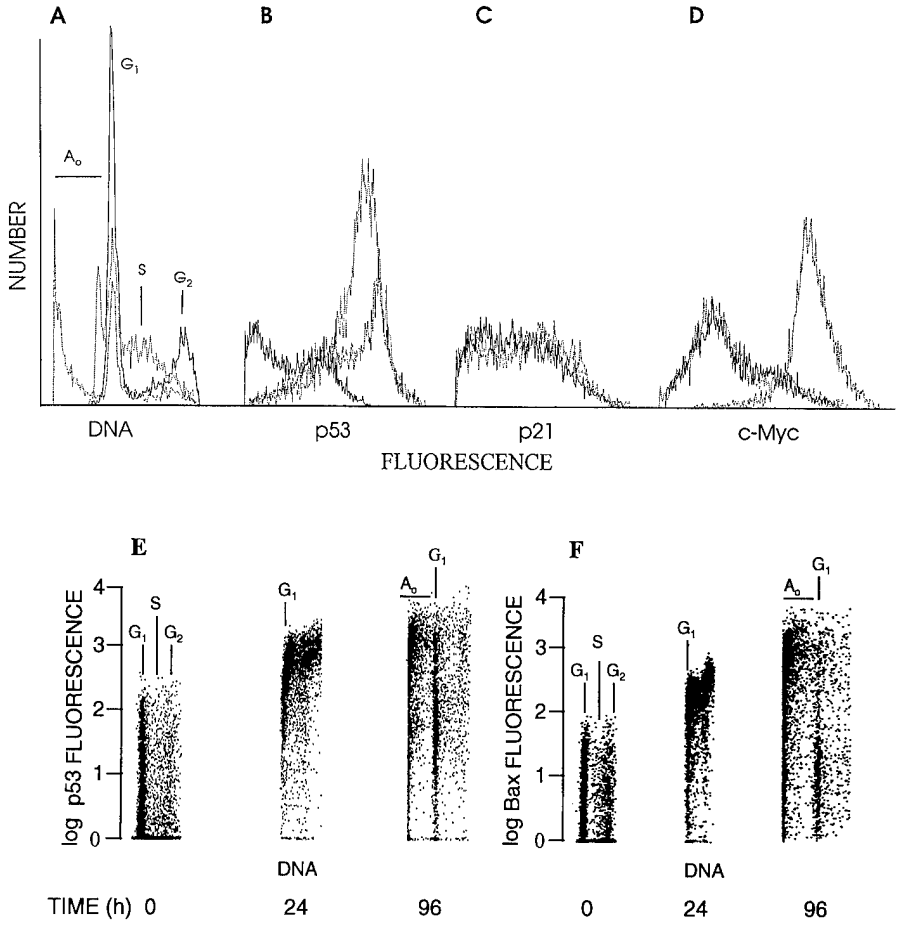

Fig. 6. OCT-Induced Apoptosis in CHO-K1 Cells Expressing hSSTR3 Is Dependent on p53, but not c-Myc

Cells were incubated with OCT (100 nM) for 0 h (*solid lines*), 24 h (*dotted lines*), and 96 h (*dash-dot-dot-dash lines*) before analysis (n = 4). The PI fluorescence in A is depicted on a linear scale, whereas p53, p21, and c-Myc immunofluorescences (B–D) are depicted on a log scale. A, OCT induced a decrease in the G_1 peak with the concomitant appearance of hypodiploid peak (A_o) within 24 h. By 96 h, cells exhibited a further decrease in G_1 and an increase in A_o. B, Induction of p53 in response to OCT treatment for 24 h is demonstrated by a net increase in both content as well as fluorescence intensity. Prolonged treatment causes a further increase in fluorescence intensity, although there is a decrease in p53. C, The p21 level does not change in response to OCT treatment. D, Induction of c-Myc in response to OCT is seen only after prolonged treatment. Flow cytometry after labeling with anti-c-Myc antibody in cells treated with 100 nM peptide for 24 h reveals no change in c-Myc compared with the control value. However, a significant increase in c-Myc fluorescence intensity as well as peak size was seen after 96 h. E, Bivariate analysis of dual labeled cells clearly shows the high p53 content of cells undergoing apoptosis (region A_0). F, Bivariate analysis of cells dual labeled with PI and anti-Bax antibody reveals that OCT induced p53-associated apoptosis is accompanied by the induction of Bax.

labeled cells showed high levels of Bax in cells characterized by hypodiploid DNA content. The slower time course of increase in Bax compared with that of wt p53 during OCT treatment suggests that the induction of Bax is a late event in hSSTR3-signaled apoptosis (details not shown). These findings are in accord with the concept that Bax is a downstream modulator of wt p53-induced apoptosis (22, 23).

The mechanism by which SSTR3 signals dephosphorylation-dependent conformational change and activation of p53 remains to be elucidated. In analogy with the activation of Ser/Thr kinases after activation of growth factor receptor tyrosine kinases, regulation of Ser phosphatase(s) may follow the SST-induced activation of PTP, which has been implicated in abrogating growth factor-induced mitogenic activity (7, 8). Inhibition of mitogenic signaling coupled with induction of wt p53 by OCT may thus facilitate the induction of apoptosis in these cells. PTP-dependent antiproliferative action of SST signaled through other SSTR subtypes may result in G_1 arrest in a p53-independent manner, as these do not transduce signals that either activate wt p53 or induce apoptosis. In contrast to the earlier claim of direct activation of PTP by SST (7), our recent findings in MCF-7 human breast adenocarcinoma cells indicate that OCT does not activate PTP in cell membranes, but induces a net increase in membrane-associated PTP in cells preincubated with the

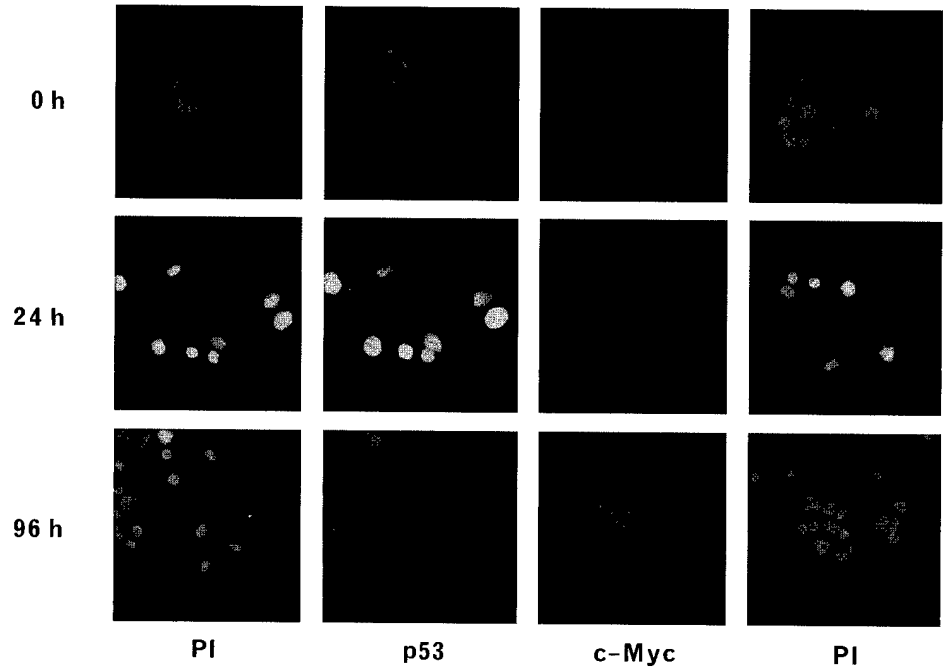

Fig. 7. Morphological Evidence of Time-Dependent Cell Shrinking and Differential Induction of p53 and c-Myc in CHO-K1 Cells Expressing hSSTR3 during Treatment with OCT (100 nM)

Aliquots of cells dual labeled with PI and p53 or c-Myc antibodies (prepared for flow cytometry) were cytospun onto microscope slides, mounted using Immunomount (Shandon, Pittsburgh, PA), viewed, and photographed through a Reichert Polyvar 2 fluorescence microscope (original magnification, ×400). Note the distinct perinuclear localization of p53 in control cells in contrast to its intranuclear localization in OCT-treated cells.

peptide (24). We have identified PTP1C as the principal tyrosine phosphatase recruited by OCT to the membrane from the cytosol in MCF-7 cells (24). It remains to be determined whether PTP1C mediates phosphorylation-dependent regulation of SST-signaled apoptosis through hSSTR3. Activation of PTP is also reported to be signaled via SSTR1 and -2 (9, 10). The present finding that neither induction of wt p53 nor apoptosis is signaled through these SSTRs raises the question of whether different PTP(s) may be involved in eliciting growth arrest in cells expressing SSTR1 and SSTR2. This remains to be clarified in future studies.

The antiproliferative action of OCT in tumor cells has been investigated in pituitary and breast cancer cells. It was reported to induce a partial G_0/G_1 block, but no apoptosis, in GH_3 rat pituitary tumor cells, in contrast to a transient G_2/M block as well as apoptosis in MCF-7 cells (4, 6, 25). We have shown that in AtT-20 mouse pituitary tumor cells, OCT induces apoptosis only in phase-synchronized, cycling cells and does not inhibit cell cycle progression in either G_0/G_1 or G_2/M (5). These tumor cells express multiple SSTR subtypes, with a predominance of SSTR1, -2, and -5, but not of SSTR3 (26, 27). Whether failure to detect apoptosis in GH_3 cells is due to a low abundance of SSTR3 and/or to the presence of mutant p53 that cannot assume wt conformation remains to be determined. The present finding that apoptosis is signaled solely through hSSTR3 coupled with our previous observation that OCT-induced apoptosis can be detected in phase-synchronized, but not asynchronous, cultures of AtT-20 cells (8) raise the possibility that this

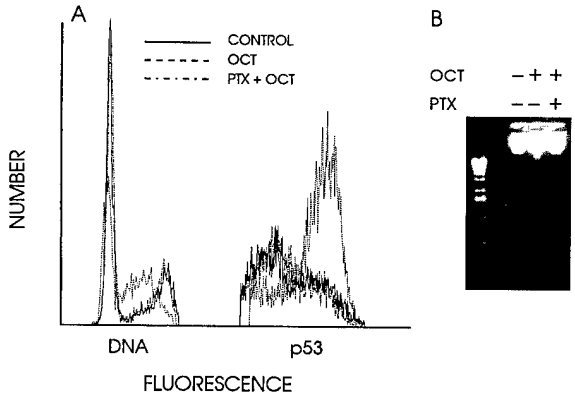

Fig. 8. Pertussis Toxin (PTX) Treatment Abolishes hSSTR3-Mediated Antiproliferative Signaling

CHO-K1 cells expressing hSSTR3 were preincubated in the presence or absence of 100 nM PTX for 18 h before the addition of 100 nM OCT. A, PTX abolishes OCT-induced changes in cell cycle parameters measured by PI staining and the increase in wt p53 detected with the anti-p53 antibody pAb 1801. PI fluorescence measurements was recorded on a linear scale (DNA), whereas p53 fluorescence was recorded on a log scale (p53). B, Representative gel demonstrating that PTX abolishes OCT-induced DNA fragmentation.

subtype may be expressed or activated preferentially in cycling cells expressing multiple SSTRs, most likely beyond the G_1 check point and in S phase, where the apoptotic process is known to be initiated (28).

In conclusion, our findings demonstrate that induction of apoptosis by OCT is selectively signaled via hSSTR3, but not other hSSTR subtypes, in CHO-K1 cells. The onset of apoptosis is preceded by induction of wt p53, which does not induce cell cycle arrest. These findings suggest that the use of SST analogs with selectivity for hSSTR3 should be of greater therapeutic value in the treatment of tumor cells that express multiple SSTRs and wt p53.

MATERIALS AND METHODS

Materials

The SST analog SMS 201–995 (OCT) was obtained from Sandoz Pharma (Basel, Switzerland). PI was purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies against p53 [pAb 1801 (wt p53 specific) and pAb 240 (mutant p53 specific)] and fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG antibody was obtained from Zymed Laboratories (San Francisco, CA). Anti-c-Myc antibody (pAb 2, monoclonal) was purchased from Oncogene Sciences (Cambridge, MA). Anti-p21 (C-19, rabbit polyclonal) antibody and anti-Bax (P-19, rabbit polyclonal) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines

Genomic fragments of hSSTR2, -3, and -5 or complementary DNA clones for hSSTR1 and -2A containing the entire coding sequences were subcloned into the polylinker region of the mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA), transfected into CHO-K1 cells, and selected for neomycin resistance as previously reported (17, 18). Cells were grown in T75 flasks in Ham's F-12 medium containing 5% FCS (Life Technologies, Gaithersburg, MD) and 400 U/ml G-418 and cultured for 3–5 days at 37 C in a humidified atmosphere with 5% CO_2. When the cells had reached 60–70% confluence, medium containing OCT at the indicated concentration was added, and the incubation was continued for different times up to 96 h. The medium containing fresh peptide was replenished every 24 h during time-course studies. The cells were then washed in PBS, scraped, and fixed first in 1% paraformaldehyde and then in 70% ethanol. Cellular DNA was labeled with the intercalating dye PI (50 mg/ml) in PBS and incubated at 37 C for 5 min in the presence of ribonuclease A (50 mg/ml). The p53 was labeled with the monoclonal anti-p53 antibody mAb 1801 or mutant-specific antibody pAb 240. c-Myc, p21, and Bax were labeled with the specific antibodies listed under *Materials*. Antigen-bound primary antibodies were then stained with FITC-conjugated goat antimouse IgG.

Flow Cytometry

Flow cytometry was carried out in an EPICS 750 series flow cytometer (Coulter Electronics, Hialeah, FL). Fluorescence was excited by a 5-watt argon laser generating light at 351–363 nm, PI emission was detected through a 610-nm long pass filter, and FITC fluorescence was detected with a 560-nm short pass dichroic filter. At least 10,000 gated events were recorded for each sample, and the data were analyzed by Winlist software (Verity Software House, ME).

Immunoblotting

Cells were lysed in Tris-HCl buffer (100 mM; pH 7.2) containing 300 mM NaCl, 2% Nonidet P-40, 20% glycerol, 2 mM $ZnCl_2$, 10 mg/ml pepstatin, and 0.2 mM Pefabloc (Boehringer Mannheim, Canada). Thirty-milligram aliquots were electrophoresed in a 10% SDS-polyacrylamide gel, transferred to Protrans Plus membranes, and probed with the anti-p53 antibody pAb 1801. Detection was carried out with an alkaline phosphatase conjugate detection kit (Bio-Rad Laboratories, Hercules, CA). Molecular size was determined using the 10-kDa protein ladder (Life Technologies) and staining with Ponceau S.

DNA Fragmentation Analysis

DNA was extracted twice with phenol-chloroform and once with chloroform from cells incubated in lysis buffer [500 mM Tris-HCl (pH 9) containing 2 mM EDTA, 10 mM NaCl, 1% SDS, and 1 mg/ml proteinase K] at 48 C for 30 h. DNA extracts were incubated with 300 mg/ml bovine pancreatic ribonuclease A at 37 C for 1 h, and 10-mg aliquots of DNA samples containing 10 mg/ml ethidium bromide were subjected to inversion field gel electrophoresis on 1.2% (wt/vol) agarose gels using the Hoefer Switchback pulse controller (Hoefer Scientific Instruments, San Francisco, CA) and visualized under UV light.

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Octapeptide Somatostatin Analog SMS 201–995 Induces Translocation of Intracellular PTP1C to Membranes in MCF-7 Human Breast Adenocarcinoma Cells*

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ABSTRACT

Somatostatin (SST) analogs exert direct antiproliferative actions in pancreatic, pituitary, and mammary tumor cells *in vitro*. SST receptor (SSTR)-mediated induction of membrane-associated protein tyrosine phosphatase (PTP) activity has been implicated in its antiproliferative signaling by virtue of its ability to dephosphorylate and inactivate growth factor receptor kinases. Recently, a PTP-containing Src homology 2 domain, identified as PTP1C/SHPTP1/SHP/HCP, was found to be associated with SSTR in rat pancreatic acinar cell membranes. In the present study we investigated the antiproliferative action of the octapeptide SST analog SMS 201–995 (OCT) and its effect on PTP activity in MCF-7 human breast adenocarcinoma cells. We report here that OCT does not directly stimulate membrane-associated PTP activity, but induces translocation of intracellular PTP to the membrane in MCF-7 cells preincubated with the peptide in a time- and concentration-dependent manner. We demonstrate that this is due at least in part to OCT-induced recruitment of cytosolic PTP1C. OCT-induced recruitment of PTP1C to the cell surface as well as its ability to inhibit the growth of MCF-7 cells was G protein dependent and inhibited by orthovanadate. These findings suggest that translocation of cytosolic PTP1C by SST analogs to the cell surface is an early event in its antiproliferative signaling in tumor cells. (*Endocrinology* **137:** 3461–3468, 1996)

REGULATION of tyrosine phosphorylation of cellular proteins plays a major role in the control of cell growth, cell differentiation, cell cycle progression, and malignant transformation (1, 2). In response to stimulation by external ligands, many cellular proteins (*e.g.* growth factor receptors and cytoplasmic kinases of the Jak and Src families) are phosphorylated on multiple tyrosine residues, resulting in immediate activation of the tyrosine kinases (3, 4). Indeed, a number of protooncogenes are tyrosine kinases, and the level of kinase activity in the corresponding oncogenes is usually several-fold higher due to constitutive autophosphorylation of tyrosine residues (5). Thus, phosphorylation on tyrosine residues, an early event in mitogenic signal transduction through growth factor receptors, can become an important factor contributing to tumorigenicity and growth of cancer cells. Inhibition of tyrosine phosphorylation has been shown to result in reduced cell proliferation, growth arrest, and cellular apoptosis (4–6). Somatostatin (SST), originally isolated as an endocrine inhibitor of pituitary GH secretion, is now recognized as a hormone capable of regulating such fundamental processes as secretion, cell division, proliferation, and apoptosis (7–12). The actions of SST are mediated by a family of specific SST receptors (SSTR) in a G protein-dependent manner and are signalled via multiple cellular effector systems, including cAMP, K^+, intracellular Ca^{2+}, phospholipase $A_2 \rightarrow$lipoxygenase, and protein tyrosine phosphatase (PTP) (for reviews, see Refs. 13 and 14). PTP induced by SST has been implicated in its antiproliferative actions by virtue of its ability to dephosphorylate and inactivate growth factor receptor kinases (15–18). Although several groups have reported modest activation of membrane-associated PTP by SST analogs, a recent study has suggested that SSTR is associated with a PTP-containing Src homology 2 (SH2) domain in rat pancreatic acinar cells (18). This PTP has been identified as PTP1C (also called SHPTP1/SHP/HCP) (18). In the present study we investigated the effect of octapeptide SST analog [SMS 201–995 (OCT), which is currently used in the treatment of tumors arising in a number of tissues, including pancreas, pituitary, and breast] on PTP activity and PTP1C in mediating its antiproliferative action in MCF-7 human breast adenocarcinoma cells. We report here that OCT does not stimulate PTP activity in MCF-7 cell membranes, but induces translocation of intracellular PTP to the cell membrane after preincubation of MCF-7 cells in a time- and concentration-dependent manner, leading to a net increase in PTP activity. This increase in PTP activity is due to OCT-induced recruitment of PTP1C to the cell surface. OCT-induced translocation of cytosolic PTP1C to the membrane and growth inhibition in MCF-7 cells occur in a G protein-dependent manner and are blocked by orthovanadate. Based on these findings, we propose that recruitment of PTP1C by OCT to the cell surface is an early event in the signal cascade leading to its antiproliferative actions.

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Materials and Methods

MCF-7, a human breast adenocarcinoma cell line, was obtained from American Type Culture Collection (Rockville, MD). The SST analog SMS 201–995 (OCT) was obtained from Sandoz (Basel, Switzerland). An al-

kaline phosphate conjugate immunodetection kit was purchased from Bio-Rad Laboratories (Hercules, CA). Pertussis toxin (islet cell-activating protein) was obtained from List Biologicals (Campbell, CA) and was activated by incubation for 10 min at 30 C in 10 mM phosphate buffer (pH 7.0) containing 5 mM dithiothreitol. All other chemicals were obtained from regular commercial sources.

Cell culture

Cells were plated in 75-cm^2 culture flasks and grown in MEM containing nonessential amino acids and supplemented with 10% FBS and 10 mg/ml bovine insulin. Medium was changed every second day until cells had reached 70–80% confluence. The cells were then incubated in the presence or absence of 10^{-12}-10^{-7} M OCT for the indicated periods of time.

Cell growth assay

Cells were plated at an initial density of 20,000 cells and grown in multiwell plates. After 24 h, the medium was changed daily, with or without the addition of 10 nM OCT. Cell growth was measured every 24 h in a Coulter counter (Hialeah, FL), and cell viability was assessed by trypan blue exclusion criteria. In parallel experiments, the effects of the addition of orthovanadate, an inhibitor of PTP, and treatment of cells with pertussis toxin, which inactivates the G proteins coupled to SSTR, were assessed.

Subcellular fractionation

Cells were washed twice in PBS and homogenized in 20 mM Tris-HCl (pH 7.2) containing 5 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM each of leupeptin and benzamidine, 0.1 mM phenylmethylsulfonylfluoride, 1 mM pepstatin A, and 10 kallikrein inhibitory units of aprotinin. After centrifugation at 800 × *g* to remove nuclear debris, the homogenate was centrifuged at 100,000 × *g* for 45 min to give a membrane pellet and a clear cytosolic supernatant. The membrane pellet was washed once and resuspended in the extraction buffer to give a protein concentration of 2 mg/ml. The protein concentration was determined according to the method of Bradford (19).

Octreotide binding to SSTRs

The [Tyr^3]OCT analog was iodinated by the chloramine-T method using carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) and purified by reverse phase HPLC on a μ-Bondapak column, as previously described (20). The specific activity of the purified [^{125}I-Tyr^3] OCT was 2000 Ci/mmol. Competitive binding assays were carried out using MCF-7 cell membranes. Thirty nanomoles of the radioligand were incubated in the absence or presence of 0–100 nM OCT with 50 μg membrane protein at 30 C for 30 min in 50 mM HEPES buffer (pH 7.5) containing 5 mM Mg^{2+}, 0.02% BSA, 200 kallikrein inhibitory units of Trasylol, and 0.02 μg/ml each of bacitracin and phenylmethylsulfonylfluoride. The membrane-associated radioactivity was separated by centrifugation, washed, and quantitated in a γ-spectrometer. The data were analyzed by the computer-assisted nonlinear regression analysis (Ligand) program.

Preparation of phosphorylated poly(Glu,Tyr)

Phosphorylated poly(Glu,Tyr) was prepared by incubating 5 mg poly(Glu,Tyr) (4:1) polymer with 2 mg phosphorylated A431 protein preparation (Upstate Biotechnology, Lake Placid, NY) in 50 mM Tris-HCl (pH 7.5) containing 0.7 mM [γ-^{32}P]ATP (700 dpm/pmol), 100 mM orthovanadate, 5 mM $MnCl_2$, and 0.1% Nonidet P-40. The reaction was allowed to proceed for 16 h at 4 C, after which the reaction mixture was fractionated on a Sephacryl S-300-HR column. Fractions containing the phosphorylated poly(Glu,Tyr) were eluted in 50 mM Tris-HCl (pH 7.5) containing 0.1% Nonidet P-40, pooled, and concentrated using a Centricon-3 microconcentrater (Amicon Corp., Danvers, MA). Aliquots of 100 μl of the concentrate were stored at −20 C.

PTP assay

PTP activity in membrane fractions was measured using [γ-^{32}P]poly-(Glu,Tyr) as substrate. Ten-microgram protein aliquots of membrane or cytosolic fractions of MCF-7 cells were incubated with 30,000 cpm [γ-^{32}P]poly(Glu,Tyr) (final concentration, 0.3 mM) at 30 C for 5 min in 50 mM Tris-HCl (pH 7) containing 1 mg/ml BSA, 5 mM dithiothreitol, and 0.2 μg/ml bacitracin in a final volume of 100 μl. The reaction was stopped by the addition of 100 ml 30% trichloroacetic acid. The mixtures were kept on ice for 30 min and centrifuged at 10,000 × *g* for 10 min to remove the denatured proteins. The inorganic phosphate ($^{32}P_i$) liberated was extracted by mixing 100 μl of the supernatant with 100 μl of a solution of 1% ammonium molybdate in 1.32 M HCl. The phosphomolybdic complex was extracted with 1 ml isobutanol-toluene (1:1), and the radioactivity was measured in a liquid scintillation counter. The amount of $^{32}P_i$ released was calculated from the specific radioactivity of [γ-^{32}P]ATP used for the phosphorylation reaction. One unit of phosphatase activity was defined as the amount of enzyme that released 1 nmol phosphate/min at 30 C from the labeled substrate. Activities of membrane-associated and cytosolic PTP in untreated control cells averaged 0.37 ± 0.06 and 0.56 ± 0.07 nmol/mg protein, respectively.

Western blotting and immunodetection of PTP1C

Membrane and cytosol fractions containing 30 μg protein were electrophoresed in a SDS-polyacrylamide gel, transferred to Protrans plus membranes, and probed with a rabbit polyclonal anti-PTP1C antibody. The generation of this antibody and its specificity for PTP1C have previously been described (21, 22). Detection was carried out with an alkaline phosphatase conjugate detection kit (Bio-Rad). Molecular size was determined using the 10-kDa protein ladder (Life Technologies, Grand Island, NY) and staining with Ponceau S.

Results

G Protein-dependent, phosphatase-mediated inhibition of MCF-7 cell growth by OCT

To study the effect of OCT on the growth of MCF-7 cells, cell proliferation was assessed during culture in the absence or presence of 10 nM peptide, with the addition of fresh medium every day. As shown in Fig. 1A, cell growth was markedly reduced by OCT over a period of 7 days. The inhibitory effect of the peptide was blocked by treatment of the cells with 100 ng/ml pertussis toxin. Likewise, sodium orthovanadate (100 μg/ml), an inhibitor of PTP, reversed OCT induced growth inhibition. The proliferation rate of these cells was not affected by either pertussis toxin or orthovanadate In a separate experiment, we investigated the inhibitory effect of OCT as a function of its concentration. The rate of cell proliferation decreased in the presence of 10^{-11}-10^{-8} M of the peptide. OCT inhibited cell growth by 50% at 93 ± 10 pM. At 10^{-7} M, OCT inhibition of cell growth was submaximal (Fig. 1B). This closely correlates with its K_i of 89 pM for SSTR binding in MCF-7 cells. In direct binding studies we confirmed the presence of high affinity SST-binding sites in MCF-7 cell membranes (K_d = 124 pM; binding capacity, 378 fmol/mg; Fig. 2).

Induction of membrane-associated PTP activity by OCT occurs in intact MCF-7 cells

As SST peptides have been reported to stimulate membrane-associated PTP in pancreatic tumor cells, we tested the effect of incubating MCF-7 cell membranes with OCT. PTP activity, measured using [γ-^{32}P]poly(Glu,Tyr) as the substrate, revealed a slight, but nonsignificant, increase in the presence of OCT over the concentration range of 10^{-11}-10^{-7}

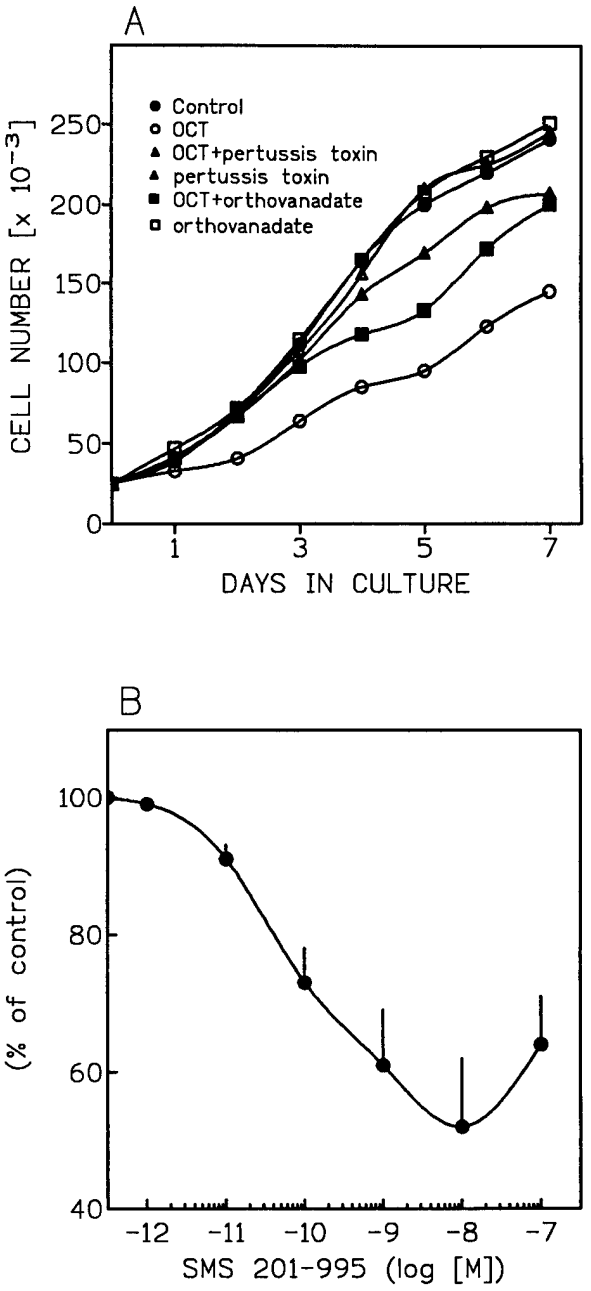


FIG. 1. Inhibition by SMS 201–995 (OCT) of MCF-7 cell proliferation. A, Cells were incubated in medium containing 10% FCS and 10 mg/ml bovine insulin in the absence or presence of 10 nM peptide alone or with the addition of 100 ng/ml pertussis toxin or 100 μg/ml orthovanadate. OCT-induced growth inhibition was evident by day 2, and maximal sustained inhibition (43 ± 7%) was seen by day 5 (*open circles*). Inclusion of pertussis toxin almost completely abolished the antiproliferative action of OCT (*closed triangles*), whereas orthovanadate significantly reversed OCT-induced growth inhibition (*closed squares*). In the absence of peptide, the cell proliferation rate was unaffected by pertussis toxin (*open triangles*) and orthovanadate (*open squares*). Values represent the cell number determined in triplicate in three separate experiments. Error bars were omitted for clarity. B, Dose dependency of the antiproliferative effect of OCT. Cell number was quantitated on day 5 after treatment with OCT (10^{-12}-10^{-7} M) in triplicate in three separate experiments. Maximum growth inhibition (43 ± 7%) occurred at 10^{-8} M OCT, whereas inhibition was less than maximal at the higher concentration of 10^{-7} M.

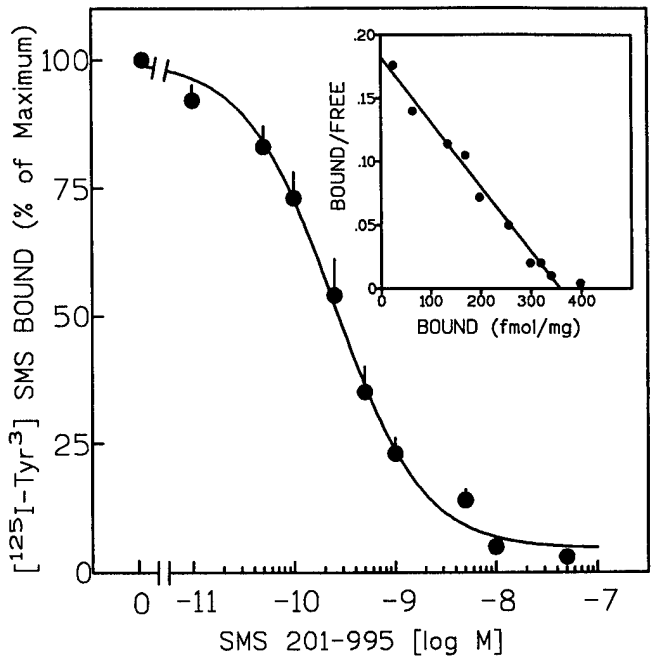


FIG. 2. Competitive inhibition by OCT of [^{125}I]Tyr^3-OCT binding to MCF-7 cell membrane SSTRs. *Inset*, Representative Scatchard plot of the competitive binding data. A single class of high affinity binding sites, with a binding capacity of 378 ± 41 fmol/mg and a K_d of 124 pM, was observed (n = 3).

M (<20% over basal activity at 10^{-7} M; Fig. 3A, *closed circles*). Likewise, OCT did not stimulate PTP activity in the cytosol when added during the enzyme assay (Fig. 3B, *closed circles*). By contrast, membrane fractions prepared from cells preincubated with the peptide for 24 h possessed markedly increased PTP activity compared with membranes of control untreated cells. Induction of membrane-associated PTP activity was clearly dependent on the concentration of peptide present in the culture medium, with half-maximal activation observed at 60 pM OCT (Fig. 3A, *open circles*). Under these conditions, OCT induced a dose-dependent decrease in the cytosolic enzyme activity (Fig. 3B, *open circles*). To determine the time course of the OCT-induced change in the subcellular distribution of PTP activity, we preincubated MCF-7 cells with 10 nM peptide from 0–24 h and analyzed the enzyme activity in the membrane and cytosolic fractions. OCT treatment of intact cells induced a rapid and prolonged increase in membrane-associated PTP activity, which was maximal (2.7-fold greater than the control value) by 4 h and remained more than 2-fold higher for up to 24 h (Fig. 3C). The increase in membrane-associated PTP was accompanied by a time-dependent decrease in cytosolic PTP activity, with a maximal 42% decrease by 4 h that was sustained for up to 24 h (Fig. 3D).

OCT induces translocation of cytosolic PTP1C to cell membrane in MCF-7 cells

PTP1C, a 66-kDa protein possessing two SH2 domains, has been shown to promote intracellular protein-protein interactions and to associate with membrane proteins (23). To test whether PTP1C is translocated to the membrane by the ac-

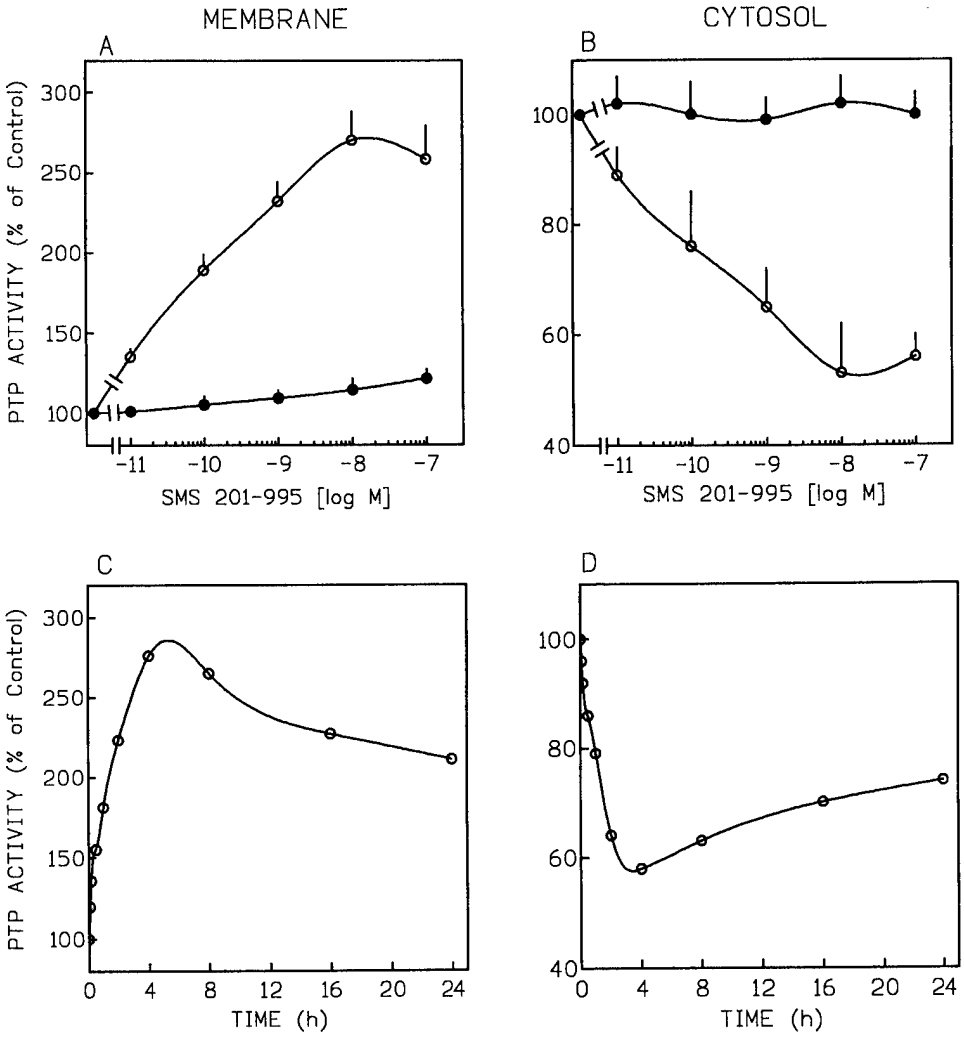


FIG. 3. OCT increases membrane-associated PTP activity in intact MCF-7 cells, but causes only minimal stimulation when incubated directly with cell membranes. A, PTP was measured in control (untreated) cell membranes incubated with OCT or in membranes of cells preincubated for 24 h with OCT. A concentration-dependent (10^{-11}-10^{-7} M) OCT-induced increase in membrane-associated PTP activity was seen in cells preincubated with OCT before subcellular fractionation (○). A maximal increase of 2.7-fold over the basal activity was seen in cells incubated with 10^{-8} M OCT. The basal activity of membranes from untreated control cells averaged 0.37 ± 0.06/mg protein. By contrast, over the same concentration range, OCT did not stimulate PTP activity when incubated with the membranes prepared from untreated MCF-7 cells (●). B, Effect of OCT on cytosolic PTP activity in MCF-7 cells. OCT induced a dose-dependent decrease in cytosolic PTP activity in cells treated with the peptide before subcellular fractionation (○), whereas no change in PTP activity was observed when OCT was incubated directly with cytosolic proteins obtained from untreated MCF-7 cells (●). C, OCT-induced increase in membrane-associated PTP activity in cells preincubated with 10 nM peptide for the indicated times. PTP activity increased by 50% within 10 min and maximally by 4 h and remained more than 2-fold higher for up to 24 h. D, OCT induced a time-dependent decrease in cytosolic PTP activity in MCF-7 cells pretreated with 10 nM OCT for the indicated times. OCT induced a rapid decrease in cytosolic PTP activity, with maximal inhibition (42%) by 4 h that was sustained for up to 24 h (mean ± SE; n = 3).

tion of OCT, Western blot analysis was performed using a rabbit anti-PTP1C antibody. In untreated MCF-7 cells, the enzyme protein exhibited preferential cytosolic localization. In cells incubated for 4 h with OCT, a concentration-dependent decrease in the intensity of this band was observed in the cytosolic fraction, with a concomitant increase in the membrane fraction (Fig. 4). The size of this band (66 kDa) was identical to that of PTP1C purified from an adenovirus expression system (21). In cells incubated with 10 nM OCT, the translocation of PTP1C to cell membrane was time dependent. The translocation occurred within minutes, and the amount of PTP1C on membranes reached a maximum by 2 h and remained elevated for up to 24 h (Fig. 5). Other SST analogs, such as RC-160, also induce redistribution of PTP1C in MCF-7 cells (24) (Srikant, C. B., and S.-H. Shen, unpublished observations). In contrast to PTP1C, OCT and other SST analogs did not induce translocation of PTP2C to the cell membrane in MCF-7 cells (data not shown). To establish whether OCT-induced augmentation of membrane-associated PTP activity is due to PTP1C, we analyzed the enzyme activity in immunoprecipitates obtained from membrane and cytosolic fractions of MCF-7 cells incubated with OCT using the PTP1C antibody. Greater than 80% of the PTP activity in the cell membrane was detected in the immuno-

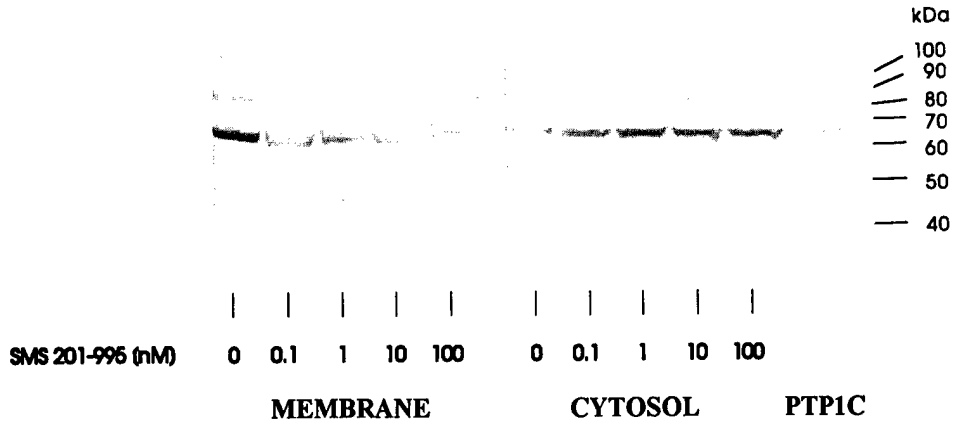
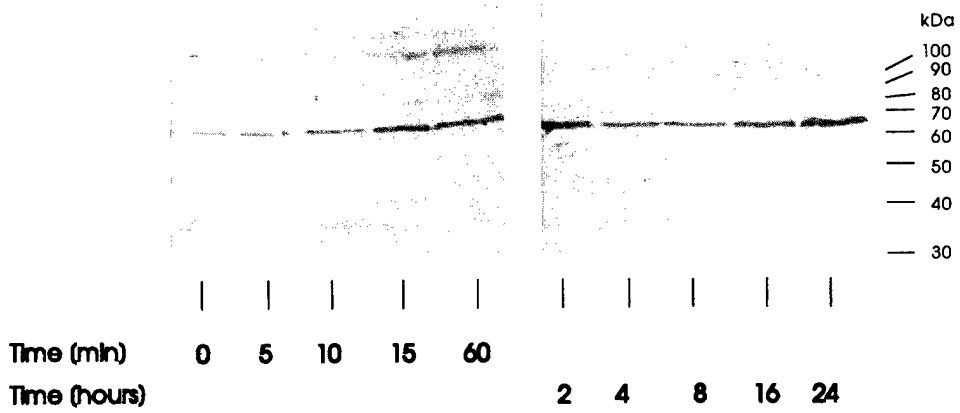


FIG. 4. Western blot analysis of PTP1C in the cytosolic and membrane fractions of MCF-7 cells preincubated with OCT. Subcellular fractions were prepared from cells incubated for 4 h with the indicated concentrations of OCT in four separate experiments, and aliquots containing 30 μg protein were electrophoresed on 10% polyacrylamide gels, transferred to Protrans membranes, and probed with anti-PTP1C antibody as described in *Materials and Methods*. As shown in this representative blot, the enzyme was detected as a 66-kDa protein, principally in the cytosol. In control cells, OCT induced a dose-dependent translocation of the intracellular enzyme to the membrane, as evidenced by the decrease in the intensity of this band in the cytosolic (*left panel*) and a concomitant increase in the membrane (*middle panel*) fractions. The size of the protein band corresponds to the size of the recombinant PTP1C purified from an adenovirus expression system (*right panel*).

FIG. 5. OCT induces a time-dependent increase in membrane-associated PTP1C. Thirty micrograms of membrane proteins prepared from cells incubated with 10 nM OCT from 5 min to 24 h were electrophoresed and analyzed as described in Fig. 4. An OCT-induced increase in membrane-associated PTP1C occurred in minutes, reached a maximum by 2–4 h, and remained elevated for up to 24 h.



precipitates, establishing that PTP1C is the principal tyrosine phosphatase translocated by the action of OCT (0.29 ± 0.09 nmol/mg in the control compared to 0.55 ± 0.13 nmol/mg in cells treated with 10^{-7} M OCT). This was further confirmed by a corresponding decrease in the activity of PTP1C immunoprecipitates obtained from cytosolic fractions of OCT (10^{-7} M)-treated cells (0.39 ± 0.13 nmol/mg in the control compared to 0.22 ± 0.07 nmol/mg in the treated cells).

OCT-induced translocation of PTP1C is inhibited by pertussis toxin and orthovanadate

To determine whether OCT-induced translocation of PTP1C to the cell membrane is G protein dependent, MCF-7 cells were pretreated for 24 h with 100 ng/ml of the toxin and then incubated with 10 nM OCT for 60 min. Pertussis toxin treatment prevented the OCT-induced translocation of cytoplasmic PTP1C to the membrane (Fig. 6, *left panel*). This was confirmed by the reversal of the OCT-induced decrease in PTP1C in the cytosolic fraction by pertussis toxin treatment of the cells (Fig. 6, *right panel*). As shown in this figure, orthovanadate, a phosphatase inhibitor, also blocked OCT-induced recruitment of PTP1C to the membrane and prevented the OCT-induced decrease in cytosolic PTP1C.

Discussion

In this study, we demonstrated that OCT, an octapeptide SST analog, binds to specific SSTRs in MCF-7 human breast cancer cells and directly inhibits cell growth in a dose-dependent manner. Pertussis toxin, which ADP ribosylates and inactivates G protein(s) linked to SSTR (for review, see Refs. 13 and 14), abolished the inhibitory effect of OCT on cell growth, suggesting that its action is G protein dependent. The fact that orthovanadate, a tyrosine phosphatase inhibitor, reversed OCT-induced growth inhibition in these cells strongly argues that PTP is involved in the SST-induced antiproliferative signal. The inhibition of cell growth by OCT described here substantiates previous reports of an antiproliferative effect of SST analogs in several tumor cells derived from breast, pancreas, pituitary, and thyroid (10–12, 15, 17, 25–31). The present findings also strengthen the emerging

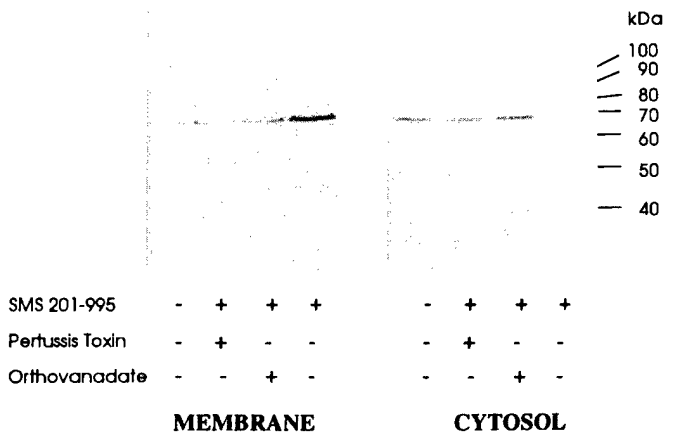


FIG. 6. OCT induced translocation of cytosolic PTP1C to the membrane in MCF-7 cells is inhibited by pertussis toxin and orthovanadate. Thirty-microgram aliquots of membrane and cytosolic fractions prepared from control cells as well as cells preincubated with 10 nM OCT for 60 min in the absence or presence of orthovanadate (100 μg/ml) or after pretreatment with pertussis toxin (100 ng/ml) were analyzed as described in Fig. 4. SMS 201–995-induced translocation of PTP1C was inhibited by both agents. This was evident from prevention of the OCT-induced increase in membrane-associated PTP1C as well as the decrease in cytosolic PTP1C.

consensus on the mediation by G protein and dependency on a SST-inducible PTP of the antiproliferative action of SST (15, 17, 31, 32).

OCT did not stimulate PTP activity in MCF-7 cell membranes, in striking contrast to the reported direct activation of PTP by SST at the membrane level in pancreatic tumor cells (17, 33). On the other hand, preincubation of the cells with the peptide induced a rapid, time- and concentration-dependent net increase in membrane-associated PTP activity. This suggests that upon binding to SSTRs, OCT acts to recruit cytosolic PTP to the cell membrane. Translocation of cytosolic PTP to the membrane in MCF-7 cells by OCT occurred rapidly, was sustained for up to 24 h, and was concentration dependent. These observations are in agreement with the finding of increased membrane-associated PTP in rat pancreatic (AR4–2J) and human colonic tumor (SW620) cells preincubated with SST analogs (34, 35). Although only a transient increase in membrane-associated PTP activity lasting 60 min or less was observed in these studies, the present data show that in MCF-7 cells preincubated with the peptide, OCT induces a sustained increase, lasting up to 24 h, in PTP activity in membrane fractions.

What is the nature of the cytosolic PTP recruited by SST to the membrane? SST-stimulated PTP was reported to be a 65- to 70-kDa protein based on gel filtration studies (36). Two members of the cytosolic PTP family have been cloned: PTP1C, which is a 66-kDa protein, and PTP2C (also known as SHPTP2/SHPTP3/PTP1D/Syp), which has a molecular size of 72 kDa (37, 38). PTP2C has been shown to function as a positive transducer in receptor tyrosine kinase-induced mitogenic signaling via the mitogen-activated protein (MAP) kinase pathway (39–41) and, as such, is unlikely to mediate the inhibitory action of SST. In studies that will be reported elsewhere, we determined that OCT does not influence the activity or the cellular localization of PTP2C in MCF-7 cells (manuscript in preparation). Therefore, PTP1C may be a suitable candidate PTP to mediate the antiproliferative signals of SST. Our finding that OCT induces translocation of PTP1C from the cytosol to the membrane in a time- and concentration-dependent manner along with its G protein dependency strengthen this idea. Thus, SST-induced translocation of cytosolic PTP1C to the membrane appears to be an early and important event in the generation of its G protein-mediated antiproliferative signals. Anionic phospholipids have been reported to increase PTP1C activity (42). The activity of PTP1C recruited to the membrane by OCT is thus likely to be regulated by anionic lipids present in the membrane environment. Such a positive regulation may explain the greater increase (2.7-fold) in membrane-associated PTP activity compared to less than 2-fold decrease in intracellular PTP. Recruitment of PTP1C to the membranes has been shown to promote association through the SH2 domains to phosphorylated epidermal growth factor and erythropoietin receptors, leading to termination of mitogenic or proliferative signals through such receptors (38, 43). We and others have shown that OCT induces apoptosis in tumor cells (11, 12). Furthermore, both pertussis toxin and orthovanadate prevent OCT-induced apoptosis in AtT-20 mouse pituitary and MCF-7 human breast tumor cells (Srikant, C. B., unpublished observations). This combined with the present finding that these agents prevent OCT-induced translocation of PTP1C in MCF-7 cells suggest a possible role for PTP1C in the signaling cascade leading to apoptosis as well. The mechanism(s) by which PTP1C may signal SST-induced apoptosis remains to be elucidated. By attenuating mitogenic signaling, SST may be expected to inhibit the MAP kinase signaling cascade. However, the reported activation of MAP kinase by SST in a heterologous cell line expressing SSTR subtype 4 raises the question of whether a positive regulation of this pathway may occur in its antiproliferative signaling (44). In this context, it is interesting to note that p38 MAP kinase activation has recently been reported to precede apoptosis induced by nerve growth factor withdrawal in PC-12 cells (45). A direct link between PTP1C and MAP kinase has not been demonstrated. It remains to be determined whether PTPs other than PTP1C play a role in SST-induced antiproliferative effects.

The inhibition of OCT-induced translocation of PTP1C by the phosphatase inhibitor orthovanadate has not previously been observed. At present, we do not know whether orthovanadate, an inhibitor of PTP, directly blocks the translocation of cytosolic PTP1C to the membrane. The possibility exists that prevention of OCT-induced translocation of PTP1C by orthovanadate may also result by an indirect mechanism dependent on its other cellular effects, such as inhibition of adenosine triphosphatase activity (46).

Five distinct SSTR subtypes, SSTRs 1–5, have been cloned from human (h), mouse (m), and rat (r) tissues (for review, see Refs. 13 and 14). The pharmacological profiles of the hSSTR subtypes expressed in Chinese hamster ovary (CHO)-K1 cells have allowed distinction of two subfamilies of SSTRs (13, 14, 47). Cyclic hexa- and octapeptide SST analogs bind to the first group, consisting of hSSTR2, -3, and -5, but they do not bind to the other subset, comprising SSTR1 and -4. It is, thus, likely that the antiproliferative action of SST peptides

signaled through PTP is mediated via SSTR2, -3, and/or -5. SST-induced growth inhibition and regulation of PTP have been investigated in CHO-DG44 cells expressing mSSTR2 and in CHO-K1 cells expressing rSSTR1, -2, and -5 and hSSTR5. SST inhibition of proliferation was seen in cells expressing all of these SSTR subtypes; increases in PTP activity were observed in cells expressing rSSTR2 and mSSTR2 and to a minor extent in cells expressing rSSTR1, but not in cells expressing rSSTR5 (31, 32, 48). The antiproliferative action of SST signaled through SSTR5 is reported to involve inhibition of Ca_i^{2+} (48). MCF-7 cells have been shown to express multiple SSTR subtypes (SSTR1, -2, -4, and -5) (49). Therefore, the antiproliferative effect of OCT in MCF-7 cells may involve inhibition of Ca_i^{2+} in addition to its recruitment of intracellular PTP1C to the cell membrane. Further studies are required to determine whether SSTR subtype- as well as agonist-selective regulation of PTP occurs by direct activation or through recruitment of intracellular PTP to the cell membrane.

In conclusion, our study demonstrates that the OCT-induced increase in membrane-associated PTP activity does not appear to result from a direct stimulation of the enzyme, but is due to recruitment of intracellular PTP to the membrane in MCF-7 breast cancer cells. This is due at least in part to OCT-induced recruitment of PTP1C to the cell membrane. OCT-induced growth inhibition as well as translocation of cytosolic PTP1C to the membrane occur in a G protein-dependent manner and are blocked by orthovanadate. We propose that recruitment of PTP1C by OCT to the cell surface is an early event in the signal cascade involved in its antiproliferative actions.

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OR39-5

EFFECTS OF GROWTH HORMONE (GH) -RELEASING FACTOR AND GH-RELEASING PEPTIDE-2 (GHRP-2) ON cAMP LEVELS, INTRACELLULAR Ca^{2+} CONCENTRATION ($[Ca^{2+}]i$) AND GH SECRETION IN HUMAN GH TUMOR CELLS

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We have shown a clear species difference in the response of sheep and rat somatotrophs to GHRP-2. GHRP-2 increased cAMP levels in sheep but not rat pituitary cells. In contrast, GHRP-6 does not stimulate cAMP production in either rat or sheep cells. The stimulation of GH secretion by GHRP-2 was totally blocked by GRF antagonist, [Tyr^1, Arg^2] GRF 1-29 but did not affect the GH release in response to GHRP-2 in rat somatotrophs. The receptor for GHRP-2 is not the GRF receptor, however, because of additive effects obtained with combined maximal doses of GRF and GHRP-2 in cultured sheep pituitary cells. In the present experiment, we examined the effects of GHRP-2 in human acromegalic tumor cells and compared the responses to those of human GRF. Acromegalic tumors were obtained at transsphenoidal adenoidectomy from 6 patients. Cells were kept in primary culture in monolayer after dissociation by collagenase. GHRP-2 induced a dose-dependent (10^{-10} to 10^{-7} M) effects on GH release from these cells up to a maximal level of 600% of control. GRF caused similar dose-dependent effects in 4/6 tumors. GRF induced a dose-dependent increase on intracellular cAMP levels from 0.4nM/10^5 cells/30 min up to 10nM/10^5 cells/30 min in all 6 tumors. GHRP-2 increased cAMP levels up to a maximum of 1.4nM/10^5 cells/30 min. The GRF and GHRP-2 -induced increase in cAMP levels was blocked by the GRF antagonist and an adenylyl cyclase inhibitor MDL (12,330A) but not by Ca^{2+} channel blockade (Co^{2+}). The PKC inhibitor (calphostin C) did not prevent the increase in cAMP levels. $[Ca^{2+}]i$ was measured using Fura-2 technique and both GRF and GHRP-2 increased $[Ca^{2+}]i$ in a dose dependent manner (blocked by Co^{2+} or Ca^{2+}-free solution. The release of GH by GRF in 4 responding tumors could be blocked by either PKA inhibitor (H89), a cAMP antagonist (Rp-cAMP), or Co^{2+} but not by calphostin C. The release of GH by GHRP-2 was not reduced by either MDL or H89 but was decreased by calphostin C and totally blocked by Co^{2+}. We conclude that GHRP-2 acts on a receptor which is different to the GRF receptor but can be blocked by GRF antagonist in human somatotrophs. Signal transduction pathways employed by GHRP-2 are different to those employed by GRF. Furthermore, in 2/6 tumors there was no GH response to GRF but an increase in cAMP levels was measured. This suggests a dissociation between increases in cAMP and GH secretion.

OR39-6

SELECTIVE MEDIATION BY HUMAN SOMATOSTATIN RECEPTOR SUB-TYPE 3 (hSSTR3) OF OCTREOTIDE REGULATION OF p53 IN CHO-K1 CELLS.

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The octreotide somatostatin analog, SMS 201-995 (OCT) inhibits tumor cell growth by both indirect (inhibition of growth factor secretion) and direct (growth arrest and/or apoptosis) mechanisms. Aberrant regulation of p53 has been reported in cells undergoing apoptosis. To examine the possibility that OCT may regulate p53 and that its effect may be mediated in a SSTR subtype selective manner, we investigated p53 in CHO-K1 cells expressing human (h) SSTR subtypes to which OCT binds (hSSTRs 2, 3 and 5). Subconfluent cells were incubated in Ham's F-12 medium supplemented with 5% fetal calf serum and antibiotics in the absence or presence of 0.1 - 100 nM OCT for 24 h. Cells were washed, fixed in 1% paraformaldehyde in PBS and 70% ethanol prior to incubating with a mouse monoclonal anti-p53 antibody (pAb1801). After washing, the cells were labeled with FITC conjugated goat anti-mouse IgG antibody. p53 labeling was quantitated by flow cytometry (Coulter Flow Cytometer) and the data analyzed using the IsoContour software (Verity Software House). 3-dimensional analysis of log FL1 (FITC fluorescence) against forward scatter indicated that OCT induced a dose-dependent increase in p53 fluorescence in a subpopulation of CHO-K1 cells expressing hSSTR3 (15% at 10^{-7} M concentration of the peptide). By contrast, p53 fluorescence in CHO-K1 cells expressing hSSTR2 and hSSTR5 was unaffected by OCT. These findings provide the first evidence for SSTR subtype-specific mediation of OCT regulation of p53, which occurs via hSSTR3. It remains to be established whether the cells exhibiting increased p53 fluorescence represent cells undergoing growth arrest or apoptosis.

OR39-7

IDENTIFICATION OF FUNCTIONAL NUCLEAR TARGETS OF SOMATOSTATIN SIGNALING PATHWAYS IN GH4 PITUITARY CELLS.

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Somatostatin (SRIF) and its analogs exert potent inhibitory effects on cellular proliferation and hormone secretion. At the level of the pituitary, SRIF has been shown to inhibit the secretion of growth hormone (GH), thyroid stimulating hormone (TSH), and under certain conditions, prolactin (PRL). These effects are mediated by a family of Gi protein-linked receptor subtypes, termed SSTRs 1-5. Despite recent progress in our understanding of SRIF action, the precise intracellular events mediating its inhibitory effects remain largely unknown. Most studies performed to date have focused on secretion and have not addressed possible effects of SRIF on pituitary gene expression. To gain a better understanding of SRIF signaling mechanisms in the pituitary, we developed a rat GH4C1 cell line stably transfected with the cDNA for the rat SSTR2, the subtype previously shown to mediate the inhibition of cAMP accumulation and GH release in pituitary cells. Several clones were derived, overexpressing varying amounts of SSTR2. BIM23014 (somatuline), a long-lasting SSTR2-selective SRIF analog, was able to block the VIP and forskolin (FSK)-induced increases in cAMP in our system, indicating that these receptors are linked to their appropriate intracellular effectors. Since many cAMP/PKA-dependent genes are ultimately regulated by the cAMP response element binding protein (CREB), we sought to determine if BIM23014 had any effects on the phosphorylation state, and hence, the transcriptional activity of this factor. SSTR2 overexpressing cells were left untreated or treated with FSK alone or FSK plus BIM23014. Western blots of whole cell extracts probed with an antibody specific for phospho-CREB indicated that the FSK-induced phosphorylation of CREB could be attenuated by pretreatment with BIM23014 to 50% of FSK only controls. As a functional correlate to these results, we then tested, utilizing a transient transfection approach, the ability of BIM23014 to inhibit the promoter activity of two relevant, pituitary-specific promoters which are known to be regulated by CREB: the human α glycoprotein subunit promoter (Hα), and the Pit-1/GHF-1 promoter. Consistent with the attenuating effects on CREB phosphorylation, BIM23014 maximally inhibited the FSK-induced stimulation of the Hα promoter 48%, and the Pit-1/GHF-1 promoter 52%, in a dose-dependent manner. Finally, BIM23014 also had partial inhibitory effects on the Pit-1/GHF-1-dependent GH and PRL promoters. These data reveal important information regarding the mechanisms of SRIF inhibition in rat pituitary cells. Specifically, the transcriptional potency of CREB is diminished by SRIF-induced attenuation of transcription factor phosphorylation. Since both CREB and Pit-1/GHF-1 govern somatotroph proliferation, these data provide mechanistic insights into how SRIF controls somatotroph cell function.

OR39-8

TRANSGENIC OVEREXPRESSION OF PITUITARY-DIRECTED LEUKEMIA INHIBITORY FACTOR: NOVEL DWARF PHENOTYPE.

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We recently demonstrated pituitary leukemia inhibitory factor (LIF) expression and LIF-mediated pituitary hormone transcription. To determine the in vivo impact of LIF expression on pituitary development and function, we established transgenic mouse lines using 320bp rat GH-promoter-driven murine LIF cDNA (670 bp) containing bGH polyA tail. Founders, screened for integration of the transgene, had low birth weights and remained from 30 to 66 % of wild type (WT) body weight for up to 10 months. Pituitaries of transgenic mice showed a 47% decrease of GH cells and the intensity of GH mRNA signal by in situ hybridization was weaker than in WT. In contrast, ACTH cell numbers increased by 79%. LIF immunoreactivity was diffuse in anterior lobe and in some cells of intermediate lobe of both transgenic and WT pituitaries. In the transgenic anterior pituitary, scattered intense LIF immunostained cells and several cystic cavities were observed; the latter were lined by cuboidal, ciliated epithelial cells, focally immunopositive for cytokeratin and S-100 and immunonegative for adenohypophysial hormones. Transgenics displayed lung congestion, diaphragmatic atrophy and males had immature germ cell-development. Serum GH levels were undetectable (< 0.78 ng/ml) in 2 different lines. Serum IGF-I levels on acid chromatographed samples (measured by Dr. Ron Rosenfeld) were 53-94 ng/ml compared to WT (215-321 ng/ml, $p<0.001$). Similarly IGFBP3 was attenuated in transgenic mice. PRL levels were significantly lower in transgenics than WT (5.4±0.43 vs 14.4±2.19 ng/ml, $p<0.05$) and T4 levels were normal. Because of impaired fertility in both males and females, ovarian transfer was required to sustain the lines. Conclusions: Expression of pituitary LIF transgene causes central GH and PRL deficiency, leading to diaphragmatic dysgenesis and impaired fertility. GH cells are decreased in number and size, and GH mRNA signal is reduced. The cystic cavities most likely represent primitive Rathke's cleft cysts suggesting that LIF interferes with the development, differentiation and appropriate maturation of the adenohypophysis. As somatotrophs and corticotrophs were discordantly regulated by GH-driven LIF, pituitary LIF behaves in both an autocrine and paracrine fashion to regulate pituitary differentiation.

P2-793

PHYTOESTROGENS INDUCE PROGESTERONE RECEPTOR IN THE ESTROGEN RESPONSIVE MCF-7 BREAST CARCINOMA CELL LINE. JM Marchiori*, J Alvarez, B Kessel, B Taback, DD Dooley, JF Mortola, RB Duda. Divisions of Reproductive Endocrinology and Surgical Oncology, Beth Israel Hospital, Boston, MA. 02215

We have previously reported that ginseng extract induces the estrogen-responsive genes, pS2 and progesterone receptor (PR), in the estrogen receptor (ER) positive MCF-7 breast cancer cell line. In order to compare the estrogenic effect of ginseng extract with other dietary phytoestrogens, daidzein, genistein, and equol were examined for induction of PR, a well characterized estrogen effect, in the MCF-7 breast cancer cell line. MCF-7 cells were cultured in phenol red-free medium, charcoal stripped for estrogen withdrawal. Ginseng root was boiled for 4 hours, and extracted with 6 volumes of chloroform-methanol (2:1 v/v), fractionated by column chromatography and dissolved in ethanol. Cells were cultured for 48 hours in the presence or absence of 17β-estradiol, ginseng extract, daidzein, genistein, or equol. The PR positive T47D breast carcinoma cell line was used as a positive control and the PR negative MB231 breast carcinoma line as a negative control. Protein was extracted by enzymatic lysis and subjected to SDS-PAGE followed by Western blot analysis using a specific monoclonal antibody against the PR. Control MCF-7 and MB231 cells did not show the presence of PR whereas T47D cells revealed bands at 90 kD and 120 kD, corresponding to the PR-A and PR-B forms, respectively. PR was significantly induced in the 17β-estradiol (10^{-9} M), ginseng extract-treated (60 μg/ml), genistein-treated (10^{-5} M), equol-treated (10^{-5} M) and daidzein treated (10^{-5} M) MCF-7 cell line. These data suggest that phytoestrogens found in a variety of herbal and dietary compounds may have estrogenic effects on human breast cancer cells and that their action is mediated via the estrogen receptor.

P2-794

EFFECTS OF AN ESTROGEN RECEPTOR SPLICE ISOFORM ON MAMMARY CELL FUNCTION. I Erenburg*[1], R. Mira-y-Lopez[2], L. Ossowski[1,2], and B. Schachter[1,3]. Depts. of [1]Cell Bio., [2]Medicine, and [3]Obs/Gyn., Mount Sinai Medical School, NYC, NY 10029.

Estrogen is a well established mitogen in breast cancer. The cellular activity of estrogen is mediated by a nuclear receptor (ER) that, as a dimer, regulates the expression of estrogen responsive genes. Several ER isoforms that arise by exclusion of specific exons in the ER mRNA have been identified. Awareness of these variants has led to the suggestion that aberrant expression of ER isoforms contributes to the development and progression of breast cancer.

One isoform, ERδ3, which lacks part of the DNA binding domain, is predicted to act as a dominant negative receptor. New data from our laboratory indicates that ERδ3 attenuates responses to the mitogenic signals of estrogen. Using the ER-positive MCF-7 breast cancer cells as a model, we stably expressed ERδ3 and isolated two positive clones with an ER:ERδ3 ratio of 1:1. These clones were strikingly different in their growth properties than the parental cells and the nonexpressing controls. Unlike control cells, which were stimulated by estradiol (E2) to grow in both anchorage-dependent and -independent conditions, ERδ3 clones were dramatically growth inhibited by E2. Indeed, in the presence of E2, colony formation in soft agar by ERδ3 cells was 35% that of the controls. These data support the hypothesis that ERδ3 dampens the mitogenic signals of estrogen. Effects of ERδ3 expression on other oncogenic indices, (e.g. invasion and protease production) are being assessed.

Our findings lead us to predict that loss of ERδ3 from normal breast epithelia allows for E2-stimulated hyperplasia. In fact, our initial analysis of normal mammary epithelia shows a higher level of ERδ3 mRNA compared to that observed in breast tumors and tumor cell lines. Together, our data support an important role for ERδ3 in the control of breast epithelial cell growth.

P2-795

INHIBITION OF ESTROGEN STIMULATED GROWTH OF MCF-7 HUMAN BREAST CANCER CELLS BY OCTREOTIDE IS POTENTIATED BY TAMOXIFEN AND INVOLVES RECRUITMENT OF PTP1C TO THE MEMBRANE. C.B. Srikant[1], J. Cai[1], K. Sharma[1] & [2]S.H. Shen. [1]Fraser Laboratories, McGill University and Royal Victoria Hospital, Montreal, P.Q. H3A 1A1 and [2]Pharmaceutical Sector, NRC Biotechnology Research Institute, Montreal, P.Q., Canada.

The octapeptide somatostatin analog SMS 201-995 (OCT) inhibits growth of steroid hormone sensitive breast cancer cells. Its ability to inhibit estrogen (E) induced cell growth is reported to be enhanced by the antiestrogen tamoxifen (TAM). Although OCT induced antiproliferative signaling is believed to involve stimulation of membrane associated protein tyrosine phosphatase (PTP), our recent findings suggest that OCT does not stimulate PTP in the E sensitive MCF-7 human breast cancer cells but induces a net increase in membrane associated PTP in cells pretreated with the peptide in a time and dose dependent manner. We have identified PTP1C (also called SHPTP1/HCP/SHP) as the principal cytosolic PTP thus recruited by OCT in MCF-7 cells. In this study we investigated the effect of estrogen (E) and TAM on OCT regulation of cell growth and PTP in MCF-7 cells. Following maintenance in E free medium for 7 days, cells were divided in to four groups and grown in medium containing E (10 nM) or E + TAM (10 nM) in the absence and presence of OCT. Cell growth and viability was monitored for 7 days. PTP was measured using [$^{32}\gamma$P]-poly (Glu-Tyr), and PTP1C was detected by Western blot analysis using a polyclonal PTP1C antibody. OCT inhibited the cell growth stimulated by E in a dose dependent manner, with maximal inhibition of 42 $\pm$ 6% seen at the 10 nM concentration. A rapid, time and concentration dependent increase in membrane associated PTP was observed in OCT treated cells. OCT induced growth inhibition as well as translocation of PTP1C to the cell membrane was enhanced by TAM. Treatment of cells with pertussis toxin reversed the growth inhibitory action of OCT. We conclude that G protein mediated recruitment of PTP1C to the membrane is an early event in the antiproliferative signaling of OCT in E sensitive human breast cancer cells and that its actions are potentiated by TAM.

P2-796

RELEASE OF ESTROGENIC XENOBIOTICS FROM FAT DEPOTS DURING FASTING. RM Bigsby*, EA Gize, R Steinmetz and A Caperell-Grant. Dept. of Obstetrics & Gynecology, Indiana University School of Medicine, Indianapolis, IN 46202-5198.

We have examined the estrogenic effects of DDT and ß-hexachlorocyclohexane (ß-HCH), persistent pesticide residues. Both induce proliferation and pS2 gene expression in cultured breast cancer cells (MCF-7, T47D); these effects are blocked by antiestrogen. In a cervical cancer cell line (HeLa) expression of an estrogen-responsive reporter gene is enhanced by these compounds only when the cells are co-transfected with an estrogen receptor expression vector. Additionally, DDT and ß-HCH induce growth of the uterus and vaginal epithelium in ovariectomized mice. These compounds are lipophilic and are found at high concentrations in human adipose tissues. Furthermore, fat levels of these compounds correlate to an increased risk of breast cancer. We tested whether fat stores of DDT or ß-HCH could be released in biologically significant amounts following fast-induced lipolysis. Adult ovariectomized mice were "loaded" with DDT or ß-HCH by administration of 4 daily doses at 100 mg/kg. Animals were then left untreated for 2 weeks at which time they were divided into 2 groups: fasted and fed. After a 2-day fast animals were killed and their uteri weighed. Fasted animals weighed an average 4.1 g less than fed animals. In ß-HCH-loaded animals that had been fasted the dry weight of the uterus (expressed as mg /g body weight) was 0.088 $\pm$ 0.012 vs. 0.052 $\pm$ 0.010 in fed animals ($p < 0.05$); weights in fasted and fed vehicle-loaded groups were 0.051 $\pm$ 0.003 and 0.050 $\pm$ 0.001, respectively. Percent tissue weight from water was also increased in the fasted, ß-HCH-loaded animals relative to controls; this is another indication of an estrogen-like effect. Fasting had no effect in DDT-loaded animals. Thus, fast-induced lipolysis can cause release of sufficient amounts of the xenobiotic ß-HCH to have an estrogenic effect in target tissues. This observation suggests an additional mechanism linking obesity and estrogen-responsive cancers.